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(71) Applicant: CYTOGEN CORPORATION, 201 College Road
East Forrestal Research Center, Princeton New
Jersey 08540 (US)

(72) Inventor: Goers, John Walter, 5200 Dolores Street,
Atascadero California (US)
Inventor: Lee, Chyl, 1347 Ute Road, New Brunswick New
Jersey (US)
Inventor: Siegel, Richard Charles, 117 Whiton Road,
Neschanic Station New Jersey (US)
Inventor: McKearn, Thomas Joseph, R.D. No. 3 Box 119,
New Hope Pennsylvania (US)
Inventor: King, Hurley Dalton, 1688-B Bluebird Drive,
Yardley Pennsylvania (US)
Inventor: Coughlin, Daniel James, 37-07 Hunters,
Plainsboro New Jersey (US)
Inventor: Rodwell, John Dennis, 430 Ramsey Road,
Yardley Pennsylvania (US)
Inventor: Alvarez, Vernon Leon, 187 Rice Drive,
Morrisville Pennsylvania (US)

(74) Representative: Warcoin, Jacques et al, Cabinet
Régimbeau 26, avenue Kléber, F-75116 Paris (FR)

(54) Antibody-therapeutic agent conjugates.

(57) This invention relates to antibody-therapeutic agent conjugates having a therapeutic agent covalently attached to an antibody or antibody fragment. Also described are methods for intermediates in the preparation of antibody conjugates. Therapeutic *in vivo* methods utilizing such antibody-therapeutic agent conjugates are described. Additionally novel photosensitizers suitable for use in preparing antibody-therapeutic agents are also described.

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ANTIBODY-THERAPEUTIC AGENT CONJUGATES

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1. FIELD OF THE INVENTION

5 The present invention relates to the general area
of antibody systems capable of delivering therapeutic agents
to target sites in vivo. The therapeutic agents are
covalently attached to antibodies or antibody fragments
either through linkers or by direct attachment to form
antibody conjugates. The antibody-therapeutic agent
10 conjugates substantially retain the immunospecificity and
immunoreactivity of the original antibody.

In a preferred embodiment the invention is directed
to attachment of a therapeutic agent through a linker which
15 may be either cleavable or non-cleavable. These antibody-
therapeutic agent conjugates which comprise the therapeutic
agent attached via the linker to the antibody molecule
substantially retain the immunospecificity and
immunoreactivity of the unconjugated antibody. Certain
20 embodiments of the invention include attachment via linkers
susceptible to cleavage by a proteolytic enzyme such that the
resulting conjugate retains the ability to bind antigen and
activate complement. The invention also includes attachment
via linkers susceptible to cleavage by urokinase, plasmin,
25 trypsin, a tissue plasminogen activator or other enzymes
having proteolytic activity. In all of these embodiments
cleavage of the linker promotes the release of the
therapeutic agent in an active or activatable form at the
target site.

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Another preferred embodiment of the invention
relates to attachment of certain therapeutic agents to an
antibody molecule such that the resulting conjugate is
delivered to a specific target site and the therapeutic agent

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is not released. The therapeutic agent may be activated at the target site.

5 Still another preferred embodiment relates to the attachment of an enzyme to an antibody molecule such that the resulting conjugate is delivered to a specific target site where the enzyme catalyzes reactions of therapeutic value.

10 The invention also relates to several methods for preparing such antibody-therapeutic agent conjugates, intermediates which are useful in preparing the conjugates, novel therapeutic agents, and methods for using such therapeutic agents..

15 2. BACKGROUND OF THE INVENTION

A variety of carrier molecules have been utilized with limited success in the delivery of therapeutic agents to a site of action. In practice the carrier should be non-toxic and target site specific. Ideally there should be a mechanism for maintenance or release of the active form of the therapeutic agent from the carrier at the target site.

2.1. CARRIER SYSTEMS

25 A number of agents have been utilized as carrier molecules with limited success in drug delivery systems. In practice the carrier should be non-toxic and target site specific. Ideally there should be a mechanism for release of the active form of the drug from the carrier at the target site. Carrier molecules such as DNA, liposomes, proteins, steroid hormones and antibodies (whole antibody molecules or fragments) have been used in conjunction with a broad spectrum of pharmaceutical or cytotoxic agents such as: radioactive compounds (e.g., ^{125}I , ^{131}I); agents which bind

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DNA, for instance, alkylating agents or various antibiotics; antimetabolites such as methotrexate; agents which act on cell surfaces (e.g., venom phospholipases and microbial toxins); and protein synthesis inhibitors (e.g., diphtheria toxin and toxic plant proteins).

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A number of investigators have reported target systems involving attachment of compounds or pharmaceutical agents directly to conventional antibodies, monoclonal antibodies, or to Fab portions of antibodies directed against tumor antigens. See Blythman et al., 1981, *Nature* 290:145-146; Davis and Preston, 1981, *Science* 213:1385-1388; Hurwitz et al., 1979, *Int. J. Cancer* 24:461-470; U.S. Patent No. 4,093,607; and U.K. Patent No. 1,446,536. Urdal and Hakomori (1980, *J. Biol. Chem.* 255(21):10509-10579) describe an antibody targeted, avidin mediated, drug killing of tumor cells.

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Although antibody carrier systems can be highly specific for the target site, a significant problem exists in that the therapeutic agent may not be released at that site. If release is necessary, the antibody-drug conjugates must be internalized by the tumor cell. There, release would occur through cleavage by lysosomal enzymes. Additionally, the non-site specific linkage of the therapeutic agent to (random) sites on the antibody molecule may interfere with antigen binding capacity, thus reducing the effectiveness of the system.

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2.2. COVALENT ATTACHMENT TO ANTIBODIES

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A number of different reactions have been used to covalently attach compounds to antibodies. This has been accomplished by reaction of the amino acid residues of the antibody molecule, including the amine groups of lysine, the

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free carboxylic acid groups of glutamic and aspartic acid, the sulfhydryl groups of cysteine and the various moieties of the aromatic amino acids.

5 There are serious disadvantages to these methods of
covalent attachment to the polypeptide backbone of an
antibody molecule. The amino acid sequences of the light and
heavy chains of immunoglobulins contain all of the amino
acids relatively regularly and randomly dispersed throughout
10 the molecule, including the antigen binding region. To the
extent any chemical modification occurs in this antigen
binding region, one has introduced a change in the
recognition element of the antibody. Such changes would be
expected to, and, in fact do, change the affinity and
specificity of the antibody for antigen. In a population of
15 different antibodies, such alteration in the antigen binding
region results in complete inactivation of some antibodies
and in lesser degrees of inactivation of others in relation
to the proximity of the alterations to the antigen binding
site. This inactivation may be due to a change within or
20 very near the antigen binding site to alter the conformation
of the binding site so as to make it unreactive, or may be
due to a change in a region outside the antigen binding
region so as to limit access of antigen to the antigen
binding region. Methods involving amino acids which are
25 relatively regularly and randomly dispersed throughout the
antibody are referred to as non-site specific methods.

 One of the most commonly used non-specific (random)
methods of covalent attachment is the carbodiimide reaction
30 to link a carboxy (or amino) group of a compound to amino (or
carboxy) groups of the antibody. Additionally, bifunctional
agents such as dialdehydes or imidoesters have been used to
link the amino group of a compound to amino groups of the
antibody molecule.

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Some investigators have used the Schiff base reaction to link compounds to antibody molecules. This method involves the periodate oxidation of a drug or cytotoxic agent that contains glycol or hydroxy groups, thus forming an aldehyde which is then reacted with the antibody molecule. Attachment occurs via formation of a Schiff base with amino groups of the antibody molecule. Isothiocyanates have been used as coupling agents for covalently attaching compounds to antibodies. By this method fluorescent compounds have been attached to antibody molecules for use in fluorescence microscopy (Brandtzaeg, 1973, Scand. J. Immunol. 2:273-290) and cell sorting systems (Loken and Herzenberg, 1975, Annals N.Y. Acad. Sci. 254:163-171).

Interchain disulfide bonds can also be used as sites of covalent attachment. However, even if one is successful in selectively reducing only the interchain disulfide bonds, several functional properties of the antibody may be adversely affected, such as functional affinity, agglutination ability and the ability to fix complement.

2.3. CARRIER SYSTEMS USING MONOCLONAL ANTIBODIES

Monoclonal antibodies produced by the hybridoma technique of Kohler and Milstein (1975, Nature 256:495-497; 1976, Eur. J. Immunol. 6:511-519) or related techniques provide distinct advantages for use as carriers for delivery of therapeutic agents to a site of action. First, monoclonal antibodies bind to only one molecular site (i.e., an epitope) with specific binding constants. Second, such antibodies are homogeneous and thus are purified with relative ease. Third, monoclonal antibodies can be made in large quantities by particular hybridoma cell lines.

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5 The discovery of tumor-produced or tumor-associated antigens has allowed the preparation of monoclonal antibodies which are immune-specific for solid tumors such as human colon, breast, hepatoma, melanoma and germ cell tumors (see reviews by Carrasquillo et al., 1984, Cancer Treatment Repts. 68:317-328; Kennel et al., 1984, Bio. Sci. 34:150-156).

10 For example, Gilliland et al. have demonstrated the therapeutic feasibility of using anti-colorectal monoclonal antibodies conjugated to diphtheria toxin A (1980, Proc. Natl. Acad. Sci. U.S.A. 77:4539). In in vitro cytotoxic assays, nearly all of the carcinoma cells treated with this conjugate were killed.

15 2.4. PHOTORADIATION THERAPY

Advances in optics technology and greater understanding of photochemistry and photobiology have raised interest in the technique of photoradiation therapy for treating a variety of disease states. Photoradiation therapy using "hematoporphyrin derivative" (HpD) for photo-
20 sensitization of tumor cells has been under development with some success for several years. Using HpD, patients have been treated for cancers such as primary and metastatic skin cancers, lung, trachea, esophagus, bladder, brain, eye
25 cancers and internal metastasis in the peritoneal cavity.

When light of the appropriate wavelength interacts with HpD, a cytotoxic mediator (i.e., the singlet oxygen) initiates chemical reactions which destroy tumor cells.
30 Indeed, HpD seems to be preferentially taken up and retained by many tumors compared to adjacent tissues (see Dougherty, In Porphyrin Photosensitization, New York: Plenum Publishing Corp., 1983, pp. 3-13). In many cases it is possible to produce a selective effect on the tumor by exposing the tumor
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and surrounding area to light of the appropriate wavelength using a laser. The laser output beam may be connected to optical fibers of the appropriate size and applied either directly into the tumor or externally to the general location of the tumor.

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Despite promising developments, however, photochemicals (e.g., hematoporphyrin and other photosensitizers) have several disadvantages for clinical use. First, there is great potential for damage to normal tissue if the areas adjacent to the tumor are not protected. There must be precise timing between drug administration and light exposure. A second disadvantage is that patients receiving photoradiation therapy are generally extremely sensitive to sunlight and must avoid exposure to the sun, frequently for as long as four weeks. Third, the dosage levels of photosensitizer required for therapy are very high and may have a negative effect on normal tissue.

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The ideal photosensitizer should be designed so that it has greater tumor specificity, requiring a lower therapeutic dose level, hence mitigating the deleterious effect of higher doses. Greater tumor specificity leads to more efficient localization to the site of action and less opportunity for dispersal throughout the body.

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Mew et al. have demonstrated the use of monoclonal antibody conjugated via carbodiimide bonding to hematoporphyrin as an anti-cancer agent in vivo and in vitro (1983, J. Immunol. 130:1473-1477).

3. SUMMARY OF THE INVENTION

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According to the general method of the present invention, a therapeutic agent is covalently attached to an

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antibody or antibody fragment. The covalent attachment of the therapeutic agent is accomplished so that the resulting antibody conjugate retains the ability to bind antigen. In particular, such methods include attachment to oxidized carbohydrate moieties of antibodies or antibody fragments, or to the sulfhydryl groups of reduced antibodies or reduced (Fab')₂ fragments.

In particular, the invention concerns methods for preparing antibody-therapeutic agent conjugates, comprising:

(a) reacting an antibody or antibody fragment with an oxidizing agent to form an aldehyde group in the carbohydrate moiety of the antibody or antibody fragment;

(b) reacting the aldehyde group of the resultant oxidized antibody or antibody fragment with an amine group of a linker, said linker containing an amine group selected from the group consisting of primary amine, secondary amine, hydrazine, hydrazide, hydroxylamine, phenylhydrazine, semicarbazide and thio-semicarbazide groups, to form an antibody-linker intermediate having substantially the same immunoreactivity and immunospecificity as the unconjugated antibody or antibody fragment; and

(c) covalently attaching the linker portion of the antibody-linker intermediate to a therapeutic agent to form an antibody-therapeutic agent conjugate.

In certain circumstances, it may be desirable to separate the above-described method for preparing antibody-therapeutic agent conjugates into two parts. The first part would produce an antibody-linker intermediate which may be considered a step in the production of the final antibody-therapeutic agent conjugate. Such antibody-linker

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intermediates may be stored for later combination with the particular therapeutic agent. Thus, the first part of the two part method would involve steps (a) and (b) above to form the intermediate antibody-linker intermediates. The second part, possibly at a later point in time, would involve covalently attaching the linker portion of the antibody-linker intermediate to a therapeutic agent to produce the final antibody-therapeutic agent conjugate.

Such antibody-therapeutic agent conjugates can also be made by alternate methods, as, for example, by first covalently attaching the linker to the therapeutic agent, and then reacting the antibody or antibody fragment with an amine group of the linker portion of the linker-therapeutic agent to form the antibody-therapeutic agent conjugate. Thus, the invention further includes a method for preparing an antibody-therapeutic agent conjugate, comprising:

(a) reacting an antibody or antibody fragment with an oxidizing agent to form an aldehyde group in the carbohydrate moiety of the antibody or antibody fragment; and

(b) reacting the aldehyde group of the resultant oxidized antibody or antibody fragment with a amine group of the linker portion of a linker-therapeutic agent intermediate, said linker-therapeutic agent intermediate, comprising a linker containing an amine group selected from the group consisting of primary amine, secondary amine, hydrazine, hydrazide, hydroxylamine, phenylhydrazine, semicarbazide and thiosemicarbazide groups, and covalently attached to a therapeutic agent, to form an antibody-therapeutic agent conjugate having substantially the same immunoreactivity and immunospecificity as the unconjugated antibody or antibody fragment.

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In either of the above embodiments, the linker may comprise a spacer element and a cleavable element. One function of the spacer element could be to position the cleavable element away from the core of the antibody molecule such that the cleavable element is more accessible to the enzyme responsible for cleavage. These embodiments would involve a method for preparing an antibody-therapeutic agent conjugate comprising:

(a) reacting an antibody or antibody fragment with an oxidizing agent to form an aldehyde group in the carbohydrate moiety of the antibody or antibody fragment;

(b) reacting the aldehyde group of the resultant oxidized antibody or antibody fragment with an amine group of a linker, said linker comprising a spacer element covalently attached to a cleavable element and said amine group located on said spacer element containing an amine group selected from the group consisting of primary amine, secondary amine, hydrazine, hydrazide, hydroxylamine, phenylhydrazine, semicarbazide and thiosemicarbazide groups, to form an antibody-linker intermediate having substantially the same immunoreactivity and immunospecificity as the unconjugated antibody or antibody fragment; and

(c) covalently attaching the cleavable element of the antibody-linker intermediate to a therapeutic agent to form an antibody-therapeutic agent conjugate.

Alternately, the above-described method for preparing antibody-therapeutic agent conjugates may be separated into two distinct parts. The first part (steps (a) and (b) above) would produce an antibody-linker intermediate which may be stored for later combination with a therapeutic agent (step (c) above).

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These antibody-therapeutic agent conjugates in which the linker comprises a spacer element and a cleavable element may also be made by first covalently attaching the linker to the therapeutic agent, and then reacting the antibody or antibody fragment with the linker portion of the linker-therapeutic agent to form the antibody-therapeutic agent conjugate. Thus, this method for preparing an antibody-therapeutic agent conjugate (having a linker comprising a spacer element and a cleavable element) comprises:

(a) reacting an antibody or antibody fragment with an oxidizing agent to form an aldehyde group in the carbohydrate moiety of the antibody or antibody fragment; and

(b) reacting the aldehyde group of the resultant oxidized antibody or antibody fragment with an amine group of a linker-therapeutic agent intermediate, said linker-therapeutic agent intermediate, comprising a spacer element covalently attached to a cleavable element and said amine group located on said spacer element and selected from the group consisting of primary amine, secondary amine, hydrazine, hydrazide, hydroxylamine, phenylhydrazine, semicarbazide and thio-semicarbazide groups, to form an antibody-therapeutic agent conjugate having substantially the same immunoreactivity and immunospecificity as the unconjugated antibody or antibody fragment.

Such antibody-therapeutic agent conjugates in which the linker comprises a spacer element and a cleavable element may be made by still other methods, for instance by first attaching the antibody to the spacer element, and then attaching the spacer element of that intermediate to a cleavable element of another intermediate comprising a

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cleavable element covalently attached to a therapeutic agent.
Such methods comprise:

5 (a) reacting an antibody or antibody fragment
with an oxidizing agent to form an aldehyde group in the
carbohydrate moiety of the antibody or antibody fragment;

10 (b) reacting the aldehyde group of the
resultant oxidized antibody or antibody fragment with a
spacer element containing an amine group selected from the
group consisting of primary amine, secondary amine,
hydrazine, hydrazide, hydroxylamine, phenylhydrazine,
semicarbazide and thio-semicarbazide groups, to form an
antibody-spacer element intermediate having substantially the
same immunoreactivity and immunospecificity as the
15 unconjugated antibody or antibody fragment; and

(c) covalently attaching the spacer element
of the antibody-spacer element intermediate to a cleavable
element of a cleavable element-therapeutic agent
20 intermediate, to form an antibody therapeutic agent
conjugate.

Still another method for preparing these antibody-
therapeutic agent conjugates involves first preparing an
25 antibody-spacer element intermediate, attaching to the spacer
element of this intermediate a cleavable element to form an
antibody-spacer element-cleavable element intermediate, and
finally attaching to the cleavable element of this
intermediate a therapeutic agent. This method comprises:

30 (a) reacting an antibody or antibody fragment
with an oxidizing agent to form an aldehyde group in the
carbohydrate moiety of the antibody or antibody fragment;

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5 (b) reacting the aldehyde group of the resultant oxidized antibody or antibody fragment with a spacer element containing an amine group selected from the group consisting of primary amine, secondary amine, hydrazine, hydrazide, hydroxylamine, phenylhydrazine, semicarbazide and thio-semicarbazide groups, to form an antibody-spacer element intermediate having substantially the same immunoreactivity and immunospecificity as the unconjugated antibody or antibody fragment;

10 (c) covalently attaching the spacer element of the antibody-spacer element intermediate to a cleavable element to form an antibody-spacer element-cleavable element intermediate; and

15 (d) covalently attaching the cleavable element of the antibody-spacer element-cleavable element intermediate to a therapeutic agent to form an antibody-therapeutic agent conjugate.

20 Other permutations of the steps of the above-described methods may be developed from knowledge of one skilled in the art and the disclosure of this specification.

25 In certain instances, another function of the spacer element could be to add multiple functional sites for subsequent attachment of cleavable elements or therapeutic agents, or cleavable element-therapeutic agent intermediates. Thus, one may attach to an aldehyde (or sulfhydryl) of the antibody molecule a "branched spacer element" having multiple
30 functional sites. Such sites may be aldehyde or sulfhydryl groups, or any chemical site to which a cleavable element, therapeutic agent or cleavable element-therapeutic agent intermediate may be attached.

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Furthermore, it can readily be seen that these same methods are applicable to instances in which the therapeutic agent is not cleavable from the antibody, that is, when there is no cleavable element in the linker. In these embodiments, the linker could be a "branched linker" having multiple functional sites for attachment directly to a therapeutic agent. Again, the functional sites may be aldehyde or sulfhydryl groups, or any chemical site to which a therapeutic agent may be attached.

In all of the above embodiments, several linkers, including branched linkers, may be attached to the same antibody molecule to form conjugates having a large number of therapeutic agents per antibody molecule.

The invention is also directed to intermediates and final products of the above-described methods. In particular, this invention encompasses antibody-linker intermediates, which comprise a linker attached via a covalent bond to a carbohydrate moiety of an oxidized antibody or antibody fragment, said antibody-linker intermediate having substantially the same immunoreactivity and immunospecificity of the original antibody or antibody fragment. In such intermediates the linkers may comprise a spacer element (attached to the carbohydrate moiety) which, in turn, is covalently attached to a cleavable element.

The invention also relates to antibody-spacer element intermediates, cleavable element-therapeutic agent intermediates and linker-therapeutic agent intermediates described in the methods above. Intermediates in which the linker is not cleavable are also encompassed by the invention.

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Further, the invention encompasses antibody-therapeutic agent conjugates which comprise a therapeutic agent covalently attached (directly or via a linker) to a carbohydrate moiety of an oxidized antibody or antibody fragment, said antibody-therapeutic agent conjugate having
5 substantially the same immunoreactivity and immunospecificity as the unconjugated antibody or antibody fragment.

Also encompassed by the invention are corresponding intermediates and antibody-therapeutic agent conjugates in
10 which the linker or therapeutic agent is attached to a sulfur atom of a reduced antibody or Fab' fragment. This embodiment of the invention involves a method for preparing an antibody-therapeutic agent conjugate, comprising:

15 (a) reacting an antibody or the (Fab')₂ fragment of an antibody with a mild reducing agent to form a reduced antibody or Fab' fragment having a sulfhydryl group;

20 (b) reacting said sulfhydryl group with a reactive group of a linker, said linker containing a reactive group selected from the group consisting of haloalkyl groups, p-mercuribenzoate groups, and groups capable of Michael-type addition reactions, to form an antibody-linker intermediate having substantially the same immunoreactivity and
25 immunospecificity as the unconjugated antibody or (Fab')₂ fragment; and

(c) covalently attaching the linker portion of the antibody-linker intermediate to a therapeutic agent to
30 form an antibody-therapeutic agent conjugate.

This method can be separated into two parts, the first part involving steps (a) and (b) above and the second

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step, separate in time, would involve producing the final conjugate as in step (c) above.

Alternatively, the same antibody-therapeutic agent conjugates can be made by another method, comprising:

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(a) reacting an antibody or the $(\text{Fab}')_2$ fragment of an antibody with a mild reducing agent to form a reduced antibody or Fab' fragment having a sulfhydryl group; and

10

(b) reacting said sulfhydryl group with a reactive group of a linker-therapeutic agent intermediate, said linker-therapeutic agent intermediate containing a reactive group selected from the group consisting of halo-alkyl groups, p-mercuribenzoate groups, and groups capable of Michael-type addition reactions, and covalently attached to a therapeutic agent to form an antibody-therapeutic agent conjugate having substantially the same immunoreactivity and immunospecificity as the unconjugated antibody or $(\text{Fab}')_2$ fragment.

20

In either of the above embodiments, the linker may comprise a spacer element covalently attached to a cleavable element. As above, the spacer-element would enable positioning of the cleavable element away from the core of the antibody molecule so that the cleavable element is more accessible to the cleaving enzyme. These embodiments involve methods comprising:

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(a) reacting an antibody or the $(\text{Fab}')_2$ fragment of an antibody with a mild reducing agent to form a reduced antibody or Fab' fragment having a sulfhydryl group;

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(b) reacting said sulfhydryl group with a reactive group of a linker, said linker comprising a spacer element covalently attached to a cleavable element and said reactive group located on said spacer element and selected from the group consisting of haloalkyl groups, p-mercuribenzoate groups, and groups capable of Michael-type addition reactions, to form an antibody-linker intermediate having substantially the same immunoreactivity and immunospecificity as the unconjugated antibody or (Fab')₂ fragment; and

(c) covalently attaching the cleavable element antibody-linker intermediate to a therapeutic agent to form an antibody-therapeutic agent conjugate.

Clearly, the above-described method may be separated into two parts, the first involving steps (a) and (b), and the second involving step (c) above.

These antibody-therapeutic agent conjugates having a linker comprising a spacer element and a cleavable element may be made by first covalently attaching the linker to the therapeutic agent, followed by reacting the reduced antibody or Fab' fragment with the linker portion of the linker-therapeutic agent to form the antibody-therapeutic agent conjugate. This method comprises:

(a) reacting an antibody or the (Fab')₂ fragment of an antibody with a mild reducing agent to form a reduced antibody or Fab' fragment having a sulfhydryl group;

(b) reacting the sulfhydryl group with a reactive group of a linker-therapeutic agent intermediate comprising a spacer element covalently attached to a cleavable element covalently attached to a therapeutic agent

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and said reactive group located on said spacer element and selected from the group consisting of haloalkyl groups, p-mercuribenzoate groups, and groups capable of Michael-type addition reactions, to form an antibody-linker intermediate having substantially the same immunoreactivity and immuno-
5 specificity as the unconjugated antibody or (Fab')₂ fragment.

These antibody-therapeutic agent conjugates having a spacer element and a cleavable element may be made by still other methods, such as by first attaching the antibody to the
10 spacer elements, and then attaching the spacer element of that intermediate to a cleavable element of another intermediate comprising a cleavable element covalently attached to a therapeutic agent. These methods comprise:

15 (a) reacting an antibody or the (Fab')₂ fragment of an antibody with a mild reducing agent to form a reduced antibody or Fab' fragment having a sulfhydryl group;

20 (b) reacting said sulfhydryl group with a reactive group of a spacer element containing a reactive group selected from the group consisting of haloalkyl groups, p-mercuribenzoate groups, and groups capable of Michael-type addition reactions, to form an antibody-spacer element intermediate having substantially the same immunoreactivity
25 and immunospecificity as the unconjugated antibody or (Fab')₂ fragment; and

(c) covalently attaching the spacer element of the antibody-spacer element intermediate to a cleavable
30 element of a cleavable element-therapeutic agent intermediate to form an antibody therapeutic agent conjugate.

Still another method for preparing these antibody-
35 therapeutic agent conjugates involves first preparing an

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antibody-spacer intermediate, followed by attaching to the spacer element of this intermediate a cleavable element to form an antibody-spacer element-cleavable element intermediate, and finally attaching to the cleavable element of this intermediate a therapeutic agent. This method
5 comprises:

(a) reacting an antibody or the $(\text{Fab}')_2$ fragment of an antibody with a mild reducing agent to form a reduced antibody or Fab' fragment having a sulfhydryl group;
10

(b) reacting said sulfhydryl group with a reactive group of a spacer element containing a reactive group selected from the group consisting of haloalkyl groups, p-mercuribenzoate groups, and groups capable of Michael-type addition reactions, to form an antibody-spacer element intermediate having substantially the same immunoreactivity and immunospecificity as the unconjugated antibody or $(\text{Fab}')_2$ fragment; and
15

(c) covalently attaching the spacer element of the antibody-spacer element intermediate to a cleavable element to form an antibody-spacer element-cleavable element intermediate having substantially the same immunoreactivity and immunospecificity as the unconjugated antibody or $(\text{Fab}')_2$ fragment; and
20
25

(d) attaching the cleavable element portion of an antibody-spacer element-cleavable element intermediate to a therapeutic agent.
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Other permutations of the order of the steps of the above-described methods may be performed by one skilled in the art based upon this disclosure.

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5 Additionally, spacer elements may have multiple functional sites for subsequent attachment of therapeutic agents, cleavable elements, or cleavable element-therapeutic agent intermediates. These functional sites may be aldehyde or sulfhydryl groups, or any chemical site to which the therapeutic agent, cleavable element or cleavable element-therapeutic agent may be attached.

10 Similarly, the above methods for attachment to sulfhydryl groups of antibodies are applicable to instances where non-cleavable linkers are employed, or where a "branched linker" is directly attached to a therapeutic agent.

15 In addition to all of these methods, the invention includes the intermediates of these methods in which the attachment is to a sulfur atom of an antibody molecule, including antibody-linker intermediates, antibody-spacer element intermediates, antibody-spacer element-cleavable element intermediates, linker-therapeutic agent
20 intermediates, cleavable element-therapeutic agent intermediates. This includes intermediates and conjugates in which the linker is not cleavable.

25 The antibody-therapeutic agent conjugates comprise a therapeutic agent covalently attached (directly or through a linker) to a sulfur atom of a reduced antibody or Fab' fragment, said antibody-therapeutic agent conjugate having substantially the same immunoreactivity and immunospecificity
30 as the unconjugated antibody or (Fab')₂ fragments.

35 The antibody-therapeutic agent conjugates of the invention are ideally suited for in vivo therapy. Delivery of therapeutic agents to specific target sites involves administering to an animal or human an effective amount of an

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antibody-therapeutic agent conjugate, wherein said antibody-therapeutic agent conjugate is immunoreactive with and immunospecific for an antigenic determinant of said specific tissue and substantially non-immunoreactive with and non-immunospecific for non-specific tissue and said antigenic determinant is not found in substantial amount in non-specific tissue.

This invention also encompasses the use of antibodies for delivery to specific cells, tissues, organs, or any other site in vivo, and the subsequent release or activation of the therapeutic agent at the target site. In one embodiment of the invention, release of the compound may be mediated by activated complement, a plasminogen activator, plasmin, a urokinase, trypsin, or another enzyme having proteolytic activity. In another embodiment of the invention, where release is not desired, photosensitive chemicals or enzymes that catalyze substrate modification with the production of cytotoxic by-products are attached to the antibody molecule.

In its most general concept, the invention contemplates site selective attachment of therapeutic agents to those areas of antibodies or antibody fragments which are not a part of nor directly involved with the antigenic site of the molecule. Thus, after selective attachment to one of these sites (located outside the antigen binding region), the antibody conjugate formed has substantially the same immunoreactivity and immunospecificity as the unconjugated antibody or antibody fragment.

Antibodies directed against any desired target (e.g., antigenic determinants of tumor cells, virus, fungi, bacteria or parasites) may be used as carrier molecules. Although conventional antibodies may be used as carrier

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molecules, monoclonal antibodies offer the advantages of increased specificity for antigen, improved efficiency of the delivery system and ease in production.

5 According to one method of the present invention, a
therapeutic agent is attached to an antibody carrier molecule
of an immunoglobulin class that is capable of complement
activation. This attachment is accomplished via linkers
10 which are susceptible to cleavage by an enzyme as enumerated
above. One or more different therapeutic agents may be
attached to each antibody molecule. The resulting antibody-
therapeutic agent conjugate is administered to an individual.
Subsequent to the binding of the antibody-therapeutic agent
15 conjugate to antigen in vivo, the individual's serum
complement is activated and the compounds will be selectively
cleaved and released at the target site.

 For release of a therapeutic agent by an enzyme
other than those of the complement system, the same linker
described supra may be attached to an antibody carrier
20 molecule of a class that does not activate complement.

 According to another method of the present
invention, a photosensitizer is attached to an antibody
carrier molecule either by a non-cleavable linker or by
25 direct attachment to the antibody molecule. After delivery
of the antibody conjugate to the target site, the photo-
sensitizer is activated by light of the appropriate
wavelength and its cytolytic effects on nearby cells are
mediated through the generation of singlet oxygen molecules
30 and oxygen free radicals.

 In an alternate embodiment of the present
invention, cleavage of the linker at the target site may not
be desirable. The linker utilized may be insensitive to
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serum proteins or the antibody molecule may be of a class or type that does not activate complement.

For the delivery of certain compounds, e.g., hormones or neurotransmitters, it may be desirable to cleave the compound without activation of the complement cascade. One may use a urokinase, tissue plasminogen activator, plasmin, trypsin or a protease-sensitive linker attached to an antibody that does or does not fix complement.

For the practice of this invention it is desirable to attach the therapeutic agent to the antibody molecule without interfering with either the antigen binding capacity of the antibody, with the ability to activate complement (also called complement fixation), or with enzyme cleavage or photoactivation of the therapeutic agent or with the process of conversion of enzyme substrates into cytotoxic by-products by the therapeutic agent. The present invention describes the novel linkers and methods of attachment which may be used to attach therapeutic agents to any antibody capable of activating complement.

4. BRIEF DESCRIPTION OF THE FIGURES

The present invention may be more fully understood by reference to the following detailed description of the invention, examples of specific embodiments of the invention and the appended figures in which:

FIG. 1 depicts a general reaction scheme for the attachment of the antineoplastic drug, Alkeran (Burroughs-Wellcome), to the peptide CBZ-gly-gly-arg.

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FIG. 2 represents Sips plots of fluorescent quenching data using unmodified antibody (●—●); antibody modified by the method of the invention (Δ—Δ); and antibody modified by attachment (carbodiimide) to aspartic and/or glutamic amino acids (□—□).

FIG. 3 represents the excitation spectra for (a) unoxidized antibody and (b) antibody oxidized in accordance with Section 7.1.

FIG. 4 represents the excitation and emission spectra of the Phenylhydrazide-Tripeptide-AMC compound prepared in accordance with Section 7.2.

FIG. 5 represents the excitation and emission spectra of the Antibody-Phenylhydrazide-Tripeptide-AMC (APTA) conjugate prepared in accordance with Section 7.3.

FIG. 6 represents the results of experiments showing the specific complement mediated release of AMC along with certain controls. Fluorescence was monitored at 460 nm with excitation at 380 nm. An increase in fluorescence indicates release of AMC from the Antibody-Phenylhydrazine-Tripeptide-AMC (APTA) conjugate; (a) represents APTA conjugate incubated with glutaraldehyde-fixed sheep red blood cells and human complement; (b) represents APTA conjugate incubated with glutaraldehyde-fixed rat red blood cells and human complement; (c) represents APTA conjugate incubated with glutaraldehyde-fixed sheep red blood cells; (d) represents APTA conjugate alone.

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5. DETAILED DESCRIPTION OF THE INVENTION

5 The present invention concerns antibody-therapeutic agent conjugates prepared by attaching a therapeutic agent to an antibody or antibody fragment directed against a target antigen. The therapeutic agent is attached either directly or via a linker to the antibody or antibody fragment. Such therapeutic agents or linkers are selectively attached to those areas of antibodies or antibody fragments which are not a part of nor directly involved with the antigen binding site of the molecule.

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5.1. ANTIBODIES

15 According to the present invention, antibodies directed against any antigen or hapten may be used. Although conventional antibodies may be used, monoclonal antibodies offer several advantages. Each monoclonal antibody is specific for one antigenic determinant. Additionally, large amounts of each monoclonal antibody can be produced.

20

Antibodies used in the present invention may be directed against any determinant, e.g., tumor, bacterial, fungal, viral, parasitic, mycoplasmal, histocompatibility, differentiation and other cell membrane antigens, pathogen surface antigens, toxins, enzymes, allergens, drugs and any biologically active molecules.

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For a more complete list of antigens, see U.S. Patent 4,193,983, particularly columns 7-11, which patent specification is incorporated herein by reference.

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Additionally, a combination of antibodies reactive to different antigenic determinants may be used.

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When delivery and release of the compound attached to the antibody are desired, immunoglobulin classes that are known to activate complement are used. In other applications, carrier immunoglobulins may be used which are not capable of complement activation. Such immunoglobulin carriers may include: certain classes of antibodies such as IgM, IgA, IgD, IgE; certain subclasses of IgG; or certain fragments of immunoglobulins, e.g., half antibody molecules (a single heavy: light chain pair), or Fab, Fab' or (Fab')₂ fragments.

Use of antibody fragments may be advantageous for delivery of therapeutic agents because these antibody fragments permeate target sites at an increased rate. The Fab' fragments of IgG immunoglobulins are obtained by cleaving the antibody molecule with pepsin [resulting in a bivalent fragment, (Fab')₂] or with papain [resulting in 2 univalent fragments, (2 Fab)]. Parham, 1983, J. Immunol. 131: 2895-2902; Lamoyi and Nisonoff, 1983, J. Immunol. Meth. 56: 235-243. The bivalent (Fab')₂ fragment can be split by mild reduction of one or a few disulfide bonds to yield univalent Fab' fragments. The Fab and (Fab')₂ fragments are smaller than a whole antibody molecule and, therefore, permeate the target site or tissue more easily. This may offer an advantage for in vivo delivery since conjugates will more readily penetrate in vivo sites (e.g., tumor masses, infection sites, etc.). An additional advantage is obtained when using conjugates formed with antibody fragments because these fragments do not cross a placental barrier. As a result, using this embodiment of the present invention, a therapeutic agent may be delivered at an in vivo site (such as a tumor) to a pregnant female without exposing the fetus to the compound.

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5.2. METHODS FOR ATTACHING THERAPEUTIC AGENTS TO ANTIBODIES AND ANTIBODY FRAGMENTS

5 The present invention utilizes several methods for
attaching therapeutic agents to antibody molecules such as
(a) attachment to the carbohydrate moieties of the antibody
or antibody fragment, or (b) attachment to sulfhydryl groups
of the antibody or antibody fragment. Whichever method is
used, the attachment must not significantly change the
essential characteristics of the antibody or antibody
10 fragment, such as immunospecificity and immunoreactivity.
Additional considerations include simplicity of reaction and
stability of the antibody conjugate produced.

15 5.2.1. ATTACHMENT TO OXIDIZED CARBOHYDRATE MOIETIES

Glycoproteins are biologically important
macromolecules which share structural characteristics
including carbohydrate residues covalently attached to a
polypeptide backbone. Since antibodies are glycoproteins,
20 compounds may be attached to the carbohydrate moiety of the
molecule. Some of the carbohydrate moieties are located on
the Fc region of the immunoglobulin and are required in order
for Cl binding to occur. The carbohydrate moiety of the Fc
region of an immunoglobulin may be utilized in the scheme
25 described herein. Alternatively, the Fab or Fab' fragments
of any immunoglobulins which contain carbohydrate moieties
may be utilized in the reaction scheme described herein. An
example of such an immunoglobulin is the human IgM sequenced
by Putnam et al. (1973, Science 182: 287).
30

As explained in detail below, the carbohydrate side
chains of antibodies or Fab or Fab' fragments may be
selectively oxidized to generate aldehydes. The resulting
aldehydes may then be reacted with amine groups (e.g.,
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ammonia derivatives such as primary amine, secondary amine, hydroxylamine, hydrazine, hydrazide, phenylhydrazine, semicarbazide or thiosemicarbazide) to form a Schiff base or reduced Schiff base (e.g., imine, enamine, oxime, hydrazone, phenylhydrazone, semicarbazone, thiosemicarbazone or reduced forms thereof).

Alternatively, the carbohydrate moiety of the antibody may be modified by enzymatic techniques so as to enable attachment to or reaction with other chemical groups. One example of such an enzyme is galactose oxidase which oxidized galactose in the presence of oxygen to form an aldehyde.

5.2.1.1. CHEMICAL METHODS OF OXIDATION

Oxidation of the carbohydrate portion or moiety of antibody molecules leads to formation of aldehyde groups. A variety of oxidizing agents can be used, such as periodic acid, paraperiodic acid, sodium metaperiodate and potassium metaperiodate. Among these, oxygen acids and salts thereof are preferred since secondary or undesirable side reactions are less frequent. For a general discussion, see Jackson, 1944, IN Organic Reactions 2, p. 341; Bunton, 1965, Oxidation in Organic Chemistry, Vol. 1 (Wiberg, ed.), Academic Press, New York, p. 367.

Oxidation of antibodies with these oxidizing agents can be carried out by known methods. In the oxidation, the antibody is used generally in the form of an aqueous solution, the concentration being generally less than 100 mg/ml, preferably 1 to 20 mg/ml. When an oxygen acid or a salt thereof is used as the oxidizing agent, it is used generally in the form of an aqueous solution, and the

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concentration is generally 0.001 to 10 mM and preferably 1.0 to 10 mM. The amount of the oxygen acid or salt thereof depends on the kind of antibody, but generally it is used in excess, for example, twice to ten times as much as the amount of the oxidizable carbohydrate. The optimal amount, however, can be determined by routine experimentation.

In the process for oxidizing antibodies with oxygen acids or salts thereof, the optional ranges include a pH of from about 4 to 8, a temperature of from 0° to 37°C, and a reaction period of from about 15 minutes to 12 hours.

During the oxidation of the glycoprotein with an oxygen acid or a salt thereof, light is preferably excluded to prevent over oxidation of the glycoprotein.

5.2.1.2. ENZYMATIC METHODS OF OXIDATION

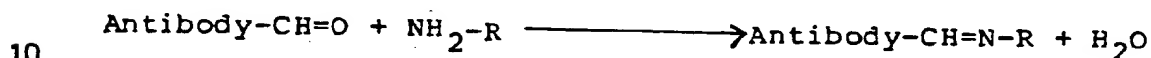
Oxidation of the carbohydrate portion of antibody molecules may also be done with the enzyme, galactose oxidase (Cooper et al., 1959, J. Biol. Chem. 234: 445-448). The antibody is used in aqueous solution, the concentration being generally 0.5 to 20 mg/ml. The enzyme generally is used at about 5 to 100 units per ml of solution, at a pH ranging from about 5.5 to about 8.0. The influence of pH, substrate concentration, buffers and buffer concentrations of enzyme reaction are reported in Cooper et al., supra.

5.2.1.3. PREPARATION OF ANTIBODY-THERAPEUTIC AGENT CONJUGATES

The antibody conjugates (or antibody linker-intermediates) of the invention may be produced by reacting

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the oxidized antibody with any linker or therapeutic agent having an available amine group selected from the group consisting of primary amine, secondary amine, hydrazine, hydrazide, hydroxylamine, phenylhydrazine, semicarbazide and thiosemicarbazide groups. The immediately resulting products contain a carbon-nitrogen double bond resulting from elimination of a molecule of water from the initial addition products:

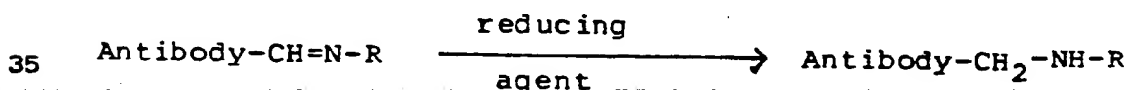


For a general discussion of the reaction of aldehydes with hydrazides, see March, 1978, IN Advanced Organic Chemistry: Reactions Mechanisms and Structure, McGraw-Hill Co., New York, pp. 824-825.

A solution of the oxidized antibody at a concentration of from about 0.5 to 20 mg/ml is mixed with the therapeutic agent or linker (molar ratios of reactive amine group to antibody aldehyde ranging from about 1 to about 10,000) and the solution incubated for from about 1 to 18 hours. Suitable temperatures are from 0 to 37°C and pH may be from about 6 to 8.

5.2.1.4. STABILIZATION OF THE ANTIBODY CONJUGATES

After the antibody-therapeutic agent conjugates (or antibody-linker intermediates) have been formed between the antibody and therapeutic agent or linker as described in Section 5.2.1.3, they can optionally be stabilized with a suitable reducing agent, such as sodium cyanoborohydride or sodium borohydride:



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Reducing agent is generally added to a molar excess of from about 10 to 100 fold molar excess over available aldehyde groups. For a general discussion, see Jentoft and Dearborn, 1979, J. Biol. Chem. 254:4359.

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5.2.2. ATTACHMENT TO SULFHYDRYL GROUPS

Free sulfhydryl groups can be generated from the disulfide bonds of the immunoglobulin molecule. This is accomplished by mild reduction of the antibody molecule. The disulfide bonds of IgG which are generally most susceptible to reduction are those that link the two heavy chains. The disulfide bonds located near the antigen binding region of the antibody molecule remain relatively unaffected. Such reduction results in the loss of ability to fix complement but does not

interfere with antibody-antigen binding ability (Karush et al., 1979, Biochem. 18:2226-2232). The free sulfhydryl groups generated in the intra-heavy chain region can then react with reactive groups of a linker or therapeutic agent to form a covalent bond which does not interfere with the antigen binding site of the immunoglobulin. Such reactive groups include, but are not limited to, reactive haloalkyl groups (including, for example, haloacetyl groups), p-mercuribenzoate groups and groups capable of Michael-type addition reactions (including, for example, maleimides and groups of the type described in Mitra and Lawton, 1979, J. Amer. Chem. Soc. 101: 3097-3110). By the term "haloalkyl" is meant any alkyl group of one to three carbon atoms substituted with bromine, iodine or chlorine.

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Details of the conditions, methods and materials suitable for mild reduction of antibodies and antibody

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fragments as described generally herein may be found in Stanworth and Turner, 1973, IN Handbook of Experimental Immunology, Vol. 1, Second Edition, Weir (ed.), Chapter 10, Blackwell Scientific Publications, London, which chapter is incorporated herein by reference.

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Antibody-therapeutic agent conjugates (or antibody-linker intermediates) which are produced by attachment to free sulfhydryl groups of reduced immunoglobulin or reduced antibody fragments do not activate complement. Thus, these conjugates may be used in in vivo systems where cleavage and release of the therapeutic agent is not desirable (e.g., where the therapeutic agent is a photosensitizer, or an enzyme that acts on a specific substrate). Such conjugates may also be used when non-complement mediated release is desired. In such an embodiment, the therapeutic agent may be linked to sulfhydryl groups on the reduced immunoglobulin or reduced antibody fragments via linkers which are susceptible to cleavage by enzymes having proteolytic activity, including but not limited to trypsin, urokinase, plasmin, tissue plasminogen activator and the like.

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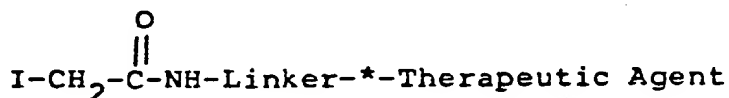
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Although attachment of a therapeutic agent to sulfhydryl groups of the antibody molecule destroys the complement fixation ability of the conjugate, such methods of attachment may be used to make antibody conjugates for use in the complement-mediated release system. In such an embodiment, a therapeutic agent joined to a complement-sensitive substrate linker can be attached to sulfhydryls of reduced IgG molecules or antibody fragments and delivered to the target in a mixture with intact antibody molecules that are capable of activating complement. The latter would activate complement which would cleave the therapeutic agent from the former. The use of antibody fragments as carrier

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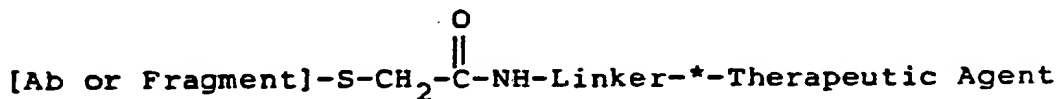
molecules in the complement mediated release system would permit the treatment of pregnant females, and offers the advantage of more rapid penetration of the conjugate into target sites.

5 According to one embodiment of the present invention, for attachment to sulfhydryl groups of reduced antibody molecules, the substrate linkers or the therapeutic agents are modified by attaching an iodoalkyl group to one end of the linker. The unmodified site on the linker may or
10 may not be covalently attached to a therapeutic agent. For instance, the substrate linkers which are ester or amide linked to therapeutic agents as prepared in Section 5.3 (see Table II and Table III) are modified by the addition of an iodoalkyl group thus forming an iodoalkyl derivative as
15 depicted below (N.B., the symbol * signifies an amide or ester bond):



20 As mentioned previously, the linker may be one that is susceptible or resistant to cleavage by activated complement, trypsin, plasmin, tissue plasminogen activator, urokinase or another specific enzyme having proteolytic activity.

25 When the iodoalkyl derivatives of the linker group are reacted with reduced antibody molecules or reduced antibody fragments, the linker group becomes covalently attached to the antibody molecules or fragment. This is
30 depicted below (N.B. the symbol * signifies as amide or ester bond):



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5.3. THERAPEUTIC AGENTS

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Antibodies may be attached to any therapeutic agent which retains its essential properties after reaction with the antibody, and which enables the antibody to
5 substantially retain immunospecificity and immunoreactivity. As used herein, the term "therapeutic agent" includes chemical modifications and derivatives of therapeutic agents which substantially retain their biological activity. The
10 major limiting factor is that any attachment reaction must be selective enough to limit competing, undesirable reactions and sufficiently mild so as not to severely interfere with antibody reactivity and selectivity.

When it is desired to attach an aldehyde of the
15 oxidized carbohydrate portion of an antibody or antibody fragment to a therapeutic agent, the therapeutic agent should contain an amine group selected from the group consisting of primary amine, secondary amine, hydrazine, hydrazide,
20 hydroxylamine, phenylhydrazine, semicarbazide and thiosemicarbazide groups. If the therapeutic agent does not contain any such amino group, the agent can be modified to introduce a suitable amine group available for coupling.

The therapeutic agent to be attached to an antibody
25 for use in a delivery system is selected according to the purpose of the intended application (i.e., killing, prevention of cell proliferation, hormone therapy or gene therapy). Such therapeutic agents may include, for example,
30 pharmaceutical agents, toxins, fragments of toxins, alkylating agents, enzymes, antibiotics, antimetabolites, antiproliferative agents, hormones, neurotransmitters, DNA, radioopaque dyes, radioactive isotopes, fluorogenic
35 compounds, marker compounds, lectins, compounds which alter cell membrane permeability, and photochemical compounds.

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Table I lists some of the pharmaceutical agents that may be employed in the herein described invention and in no way is meant to be an exhaustive list. Finally, combinations of therapeutic agents may be used.

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TABLE I
EXAMPLES OF THERAPEUTIC AGENTS FOR ANTIBODY-MEDIATED DELIVERY

	NAME/CLASS	LINKAGE	MANUFACTURERS(S)
I. ANTIBACTERIALS			
5	Aminoglycosides		
	Streptomycin	ester/amide	
	Neomycin	ester/amide	Dow, Lilly, Dome, Pfipharmics
	Kanamycin	ester/amide	Bristol
	Amikacin	ester	Bristol
	Gentamicin	ester/amide	Upjohn, Wyeth, Schering
	Tobramycin	ester/amide	Lilly
10	Streptomycin B	ester/amide	Squibb
	Spectinomycin	ester	Upjohn
	Ampicillin	amide	Squibb, Parke-Davis, Comer, Wyeth, Upjohn, Bristol, SKF
	Sulfanilamide	amide	Merrell-National
	Polymyxin	amide	Burroughs-Wellcome, Dow, Parke-Davis
	Chloramphenicol	ester	Parke-Davis
15	II. ANTIVIRALS		
	Acyclovir		Burroughs-Wellcome
	Vira A	ester/amide	Parke-Davis
	Symmetrel	amide	Endo
	III. ANTIFUNGALS		
20	Nystatin	ester	Squibb, Primo, Lederle, Pfizer, Holland-Rantor
	IV. ANTINEOPLASTICS		
	Adriamycin	ester/amide	Adria
	Cerubidine	ester/amide	Ives
	Bleomycin	ester/amide	Bristol
	Alkeran	amide	Burroughs-Wellcome
25	Valban	ester	Lilly
	Oncovin	ester	Lilly
	Fluorouracil	ester	Adria, Roche, Herbert
	Methotrexate	amide	Lederle
	Thiotepa	-	Lederle
	Bisantrene	-	Lederle
	Novantrone	ester	Lederle
30	Thioguanine	amide	Burroughs-Wellcome
	Procarabazine	-	Hoffman La Roche
	Cytarabine	-	Upjohn

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V. RADIOPHARMACEUTICALS

^{125}I
 ^{131}I
 $^{99\text{m}}\text{Tc}$ (Technetium)

VI. HEAVY METALS

5 Barium
 Gold
 Platinum

VII. ANTIMYCOPLASMAALS

 Tylosine
 Spectinomycin

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According to one embodiment of the present invention, photochemicals including photosensitizers and photothermolytic agents may be used as therapeutic agents. Efficient photosensitizers include, but are not limited to porphyrins and modified porphyrins (e.g., hematoporphyrin, hematoporphyrin dihydrazide, deuteroporphyrin dihydrazide and protoporphyrin dihydrazide), rose bengal, acridines, thiazines, xanthenes, anthraquinones, azines, flavin and nonmetal-containing porphyrins, porphyrin-like compounds, methylene blue, eosin, erythrosin, psoralin and the like. Other photosensitizers include, but are not limited to tetracyclines (e.g., dimethylchlor tetracycline) sulfonamides (e.g., sulfanilamide), griseofulvin, phenothiazines, (e.g., chlorpromazine), thiazides, sulfonylurea, and many others. Photochemicals may be designed or synthetically prepared to absorb light at specific wavelengths. Photothermolytic agents, such as Azure A, which are activated at the site of action by a light source (see Anderson and Parrish, 1983, Science 220: 524-527) may be utilized as therapeutic agents.

According to another embodiment of the present invention, enzymes that catalyze substrate modification with the production of cytotoxic by-products may be used as therapeutic agents. Examples of such enzymes include but are not limited to glucose oxidase, galactose oxidase, xanthene oxidase and the like.

5.4. LINKERS

According to the invention, antibodies may be covalently attached to a therapeutic agent through an intermediate linker having at least two reactive groups, one to react with antibody and one to react with the therapeutic agent. The linker, which may include any compatible organic

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compound, must be chosen such that the reaction with antibody (or therapeutic agent) does not adversely affect antibody reactivity and selectivity. Furthermore, the attachment of linker to therapeutic agent must not destroy the activity of the therapeutic agent. Suitable linkers for reaction with oxidized antibodies or oxidized antibody fragments include those containing an amine selected from the group consisting of primary amine, secondary amine, hydrazine, hydrazide, hydroxylamine, phenylhydrazine, semicarbazide and thiosemicarbazide groups. Such reactive functional groups may exist as part of the structure of the linker, or may be introduced by suitable chemical modification of linkers not containing such groups.

According to the present invention, suitable linkers for attachment to reduced antibodies or antibody fragments include those having certain reactive groups capable of reaction with a sulfhydryl group of a reduced antibody or Fab' fragment. Such reactive groups include, but are not limited to: reactive haloalkyl groups (including, for example, haloacetyl groups), p-mercuribenzoate groups and groups capable of Michael-type addition reactions (including, for example, maleimides and groups of the type described by Mitra and Lawton, 1979, J. Amer. Chem. Soc. 101: 3097-3110). By the term "haloalkyl" is meant any alkyl group of one to three carbon atoms substituted with bromine, iodine or chlorine.

The therapeutic agent may be attached to the linker before or after the linker is attached to the antibody molecule. In certain applications it may be desirable to first produce an antibody-linker intermediate in which the linker is free of an associated therapeutic agent. Depending upon the particular application, a specific therapeutic agent may then be covalently attached to the linker.

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5.4.1. BRANCHED LINKERS

Of additional interest are "branched linkers" which have multiple sites for attachment of therapeutic agents. For multiple site linkers, a single covalent attachment to an antibody or antibody fragment would result in an antibody-linker intermediate capable of binding a therapeutic agent at a number of sites. The sites may be aldehyde or sulfhydryl groups or any chemical site to which therapeutic agents can be attached.

Alternatively, higher specific activity (or higher ratio of therapeutic agents to antibody molecule) can be achieved by attachment of a single site linker at a plurality of sites on the antibody or antibody fragment. This plurality of sites may be introduced into the antibody or antibody fragment by either of two methods either of two methods. First, one may generate multiple aldehyde groups and/or sulfhydryl groups in the same antibody molecule. Second, one may attach to an aldehyde or sulfhydryl of the antibody molecule a "branched linker" having multiple functional sites for subsequent attachment to linkers. The functional sites of the branched linker or multiple site linker may be aldehyde or sulfhydryl groups, or may be any chemical site to which linkers may be attached. Still higher specific activities may be obtained by combining these two approaches, that is, attaching multiple site linkers at several sites on the antibody or antibody fragment.

5.4.2. CLEAVABLE LINKERS

Peptide linkers which are susceptible to cleavage by enzymes of the complement system, urokinase, tissue plasminogen activator, trypsin, plasmin, or another enzyme

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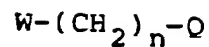
having proteolytic activity may be used in one embodiment of the present invention. According to one method of the present invention, a therapeutic agent is attached via a linker susceptible to cleavage by complement. The antibody is selected from a class which can activate complement. The antibody-therapeutic agent conjugate, thus, activates the complement cascade and releases the therapeutic agent at the target site. According to another method of the present invention, attachment of a therapeutic agent to an antibody via sulfhydryl groups destroys the ability of the antibody therapeutic agent to activate complement. If release by complement is desired, another immunoglobulin directed to the target site and capable of complement activation must be administered in combination with the antibody-therapeutic agent conjugate. According to another method of the present invention, a therapeutic agent is attached via a linker susceptible to cleavage by enzymes having a proteolytic activity such as a urokinase, a tissue plasimogen activator, plasmin, or trypsin.

In addition therapeutic agents may be attached via disulfide bonds (for example, the disulfide bonds on a cysteine molecule) to the antibody molecule. Since many tumors naturally release high levels of glutathione (a reducing agent) this can reduce the disulfide bonds with subsequent release of the therapeutic agent at the site of delivery.

5.4.3. SPACERS AND CLEAVABLE ELEMENTS

In still another embodiment, it may be necessary to construct the linker in such a way as to optimize the spacing between the therapeutic agent and the antibody. This may be accomplished by use of a linker of the general structure:

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wherein W is either $-NH-CH_2-$ or $-CH_2-$;
Q is an amino acid, peptide; and
n is an integer from 0 to 20.

5

10 In still other embodiments, the linker may comprise a spacer element and a cleavable element. The spacer element serves to position the cleavable element away from the core of the antibody molecule such that the cleavable element is more accessible to the enzyme responsible for cleavage. Certain of the "branched linkers" described above may serve as spacer elements.

15

Throughout this discussion, it should be understood that the attachment of linker to therapeutic agent (or of spacer element to cleavable element, or cleavable element to therapeutic agent) need not be particular mode of attachment or reaction. Any reaction providing a product of suitable stability and biological compatibility is acceptable.

20

5.4.4. SERUM COMPLEMENT AND SELECTION OF LINKERS

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According to one method of the present invention, when release of a therapeutic agent is desired, an antibody of a class which can activate complement is used. The resulting retains the ability to bind antigen and activate the complement cascade.

30

Complement is the collective name for a group of serum proteins which can be activated in one of two ways, the classical pathway and the properdin pathway (Müller-Eberhard, Hospital Practice, August 1977:33-43). The classical pathway is initiated by the binding of antibodies of the IgM class or certain subclasses of IgG to its corresponding antigen

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whereas the properdin pathway is dependent upon the serum protein, properdin and other non-immunoglobulin serum factors (Reid and Porter, 1981, Ann. Rev. Biochem. 50:433-464).

5 The classical pathway is a pathway of particular importance of the practice of the present invention. The classical pathway is characterized by the formation of certain antibody-antigen complexes (or immune complexes) which activate the proteolytic enzymes of the complement system (Borsos and Rapp, 1965, J. Immunol. 95:559-566; Cohen, 10 1968, J. Immunol. 100:407-413; Cohen and Becker, 1968, J. Immunol. 100:403-406; Ishizaka et al., 1968, J. Immunol. 100:1145-1153). These activated complement enzymes cleave and activate other components of the complement cascade. Ultimately the formation of an "attack complex" (or lytic 15 complex) is induced resulting in disruption of target cell membrane integrity.

The first component activated in the classical pathway is C1 which becomes a protease that acts on both C2 20 and C4. Activated C1 (C1 \bar{I}) has a specific esterase activity. Activated C4,2 (C4,2 $\bar{2}$) sometimes called C3 convertase, is a complex which proteolytically cleaves C3, and together with activated C3 (C3b), cleaves C5. Cleavage of C3 is the first step in common between the classical and properdin pathways 25 of complement activation.

The enzymatic activities of both C1 \bar{I} and C4,2 $\bar{2}$ have been studied in vitro with synthetic peptide substrates (see Table II) which are cleaved at a carboxy terminal ester or 30 amide bond. These synthetic peptide substrates may be used as linkers between an antibody molecule and a therapeutic agent as described in the present invention, or as cleavable elements of linkers having a spacer element and a cleavable element. Such linkers may allow for the specific complement 35

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mediated cleavage and subsequent release of the therapeutic agent in its active form at the target site. However, any substrate which is susceptible to cleavage by any of the components of complement, trypsin, tissue plasminogen activator, urokinase, plasmin, or any enzyme having
5 proteolytic activity may be used as a linker.

Thus, according to this embodiment of the present invention, a therapeutic agent is joined to one end of the cleavable linker or cleavable element and the other end of
10 the linker group is attached to a specific site on the antibody molecule. For example, if the therapeutic agent has a hydroxy group or an amino group, it may be attached to the carboxy terminus of a peptide, amino acid or other suitably
15 chosen linker via an ester or amide bond, respectively. For example, such agents may be attached to the linker peptide via a carbodiimide reaction. If the therapeutic agent contains functional groups that would interfere with attachment to the linker, these interfering functional groups can be blocked before attachment and deblocked once the
20 product conjugate or intermediate is made. For example, FIG. 1 depicts a general reaction scheme for the attachment of the antineoplastic drug, Alkeran (Burroughs-Wellcome) to the peptide CBZ-gly-gly-arg. The opposite or amino terminus of the linker is then used either directly or after further
25 modification for binding to an antibody molecule which is capable of activating complement.

Linkers (or spacer elements of linkers) may be of any desired length, one end of which can be covalently at-
30 tached to specific sites on the antibody molecule. The other end of the linker or spacer element may be attached to an amino acid or peptide linker. Table III lists some cleavable elements that may be used as linker groups to prepare the antibody-therapeutic agent conjugates of the present
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invention. (In the table n may be an integer including zero.) These sequences were derived from those of the complement substrate sequences by substituting amino acids with similar acid-base properties. This list is not exhaustive.

5

Thus when these conjugates bind to antigen in the presence of complement the amide or ester bond which attaches the therapeutic agent to the linker will be cleaved, resulting in release of the agent in its active form. These conjugates, when administered to an individual, will accomplish delivery and release of the therapeutic agent at the target site, and are particularly effective for the in vivo delivery of pharmaceutical agents, antibiotics, antimetabolites, antiproliferative agents and the like.

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TABLE II

SYNTHETIC SUBSTRATES FOR COMPLEMENT COMPONENTS

5

Reference No.*

For C $\bar{1}$:

	N-Boc-tyrosine o-nitrophenyl ester	1
	N-Boc-phenylalanine o-nitrophenyl ester	1
10	N-Boc-lysine o-nitrophenyl ester	1
	N-CBZ-tyrosine p-nitrophenyl ester	2

For C $\bar{4}$, 2:

	N-acetyl-gly-lys-methyl ester	3
	N-CBZ-lys-methyl ester	3
15	N-acetyl-lys-methyl ester	3
	Boc-leu-gly-arg-7-amino-4-methylcoumarin	4

- * 1. Sim et al., 1977, Biochem. J. 163:219-27.
2. Bing 1969, Biochemistry 8: 4503-10.
20 3. Cooper N.R., 1975, Biochemistry 14:4245-51.
4. Caparale et al., 1981, J. Immunol. 128:1963-65.

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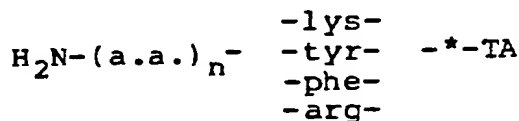
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TABLE III

LINKER GROUPS FOR ATTACHMENT OF
THERAPEUTIC AGENTS (TA) TO ANTIBODY MOLECULES¹

5

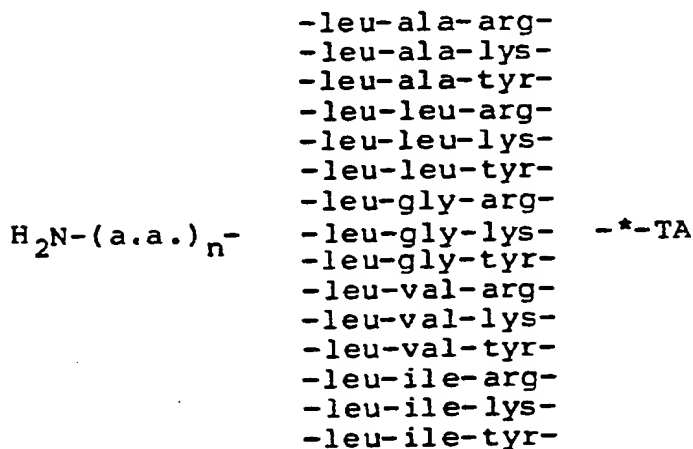
A. Linkers For Cleavage by C₁



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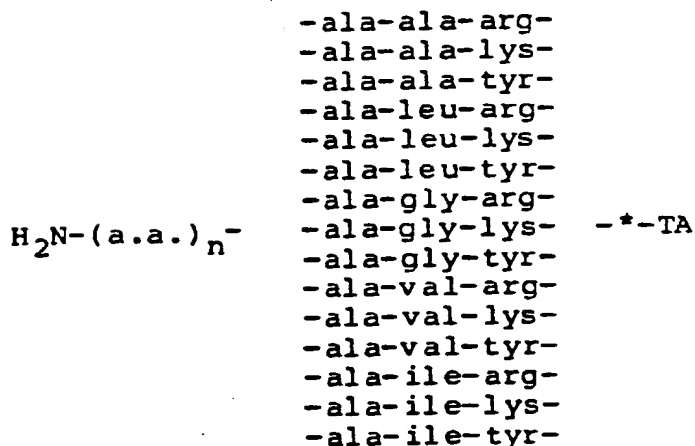
B. Tripeptide Sequences For Cleavage by C_{4,2}

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¹ The asterisk (*) represents either an amide bond
(Linker-C(=O)-NH-CI) or an ester bond (Linker-C(=O)-O-CI).

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III. B. Tripeptide Sequences For Cleavage by C4,2
(Continued)

		-gly-ala-arg-	
		-gly-ala-lys-	
		-gly-ala-tyr-	
5		-gly-leu-arg-	
		-gly-leu-lys-	
		-gly-gly-arg-	
	H ₂ N-(a.a.) _n -	-gly-gly-lys-	-*-TA
		-gly-gly-tyr-	
		-gly-val-arg-	
		-gly-val-lys-	
10		-gly-val-tyr-	
		-gly-ile-arg-	
		-gly-ile-lys-	
		-gly-ile-tyr-	
		-val-ala-arg-	
		-val-ala-lys-	
		-val-ala-tyr-	
15		-val-leu-arg-	
		-val-leu-lys-	
		-val-leu-tyr-	
		-val-gly-arg-	
	H ₂ N-(a.a.) _n -	-val-gly-lys-	-*-TA
		-val-gly-tyr-	
		-val-val-arg-	
20		-val-val-lys-	
		-val-val-tyr-	
		-val-ile-arg-	
		-val-ile-lys-	
		-val-ile-tyr-	
		-ile-ala-arg-	
		-ile-ala-lys-	
25		-ile-ala-tyr-	
		-ile-leu-arg-	
		-ile-leu-lys-	
		-ile-leu-tyr-	
		-ile-gly-arg-	
	H ₂ N(a.a.) _n -	-ile-gly-lys-	-*-TA
		-ile-gly-tyr-	
		-ile-val-arg-	
30		-ile-val-lys-	
		-ile-val-tyr-	
		-ile-ile-arg-	
		-ile-ile-lys-	
		-ile-ile-tyr-	

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III C. Peptide Sequences for Cleavage by C₄,₂

		-leu-gly-	
		-leu-leu-	
		-leu-ala-	
		-leu-val-	
5		-leu-ile-	
		-gly-gly-	
		-gly-leu-	
		-gly-ala-	
		-gly-val-	
	H ₂ N-	-ala-gly-	-Tripeptide ² -*-TA
		-ala-leu-	
10		-ala-ala-	
		-ala-val-	
		-ala-ile-	
		-val-gly-	
		-val-leu-	
		-val-ala-	
		-val-val-	
		-val-ile-	
15		-ile-gly-	
		-ile-leu-	
		-ile-ala-	
		-ile-val-	
		-ile-ile-	

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² Tripeptide represents any of the tripeptides listed in Table III B.

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5.4.5. LINKERS FOR RELEASE WITHOUT COMPLEMENT
ACTIVATION

5 In yet another application of targeted delivery,
release of the therapeutic agent without complement
activation is desired since activation of the complement
cascade will ultimately lyse the target cell. Hence, this
approach is useful when delivery and release of the
therapeutic agent should be accomplished without killing the
10 target cell. Such is the goal when delivery of cell
mediators such as hormones, enzymes, corticosteroids, neur-
otransmitters, genes or enzymes to target cells is desired.
These conjugates may be prepared by attaching the therapeutic
agent to an antibody molecule or fragment that is not capable
15 of activating complement via a linker that is mildly
susceptible to cleavage by serum proteases. When this
conjugate is administered to an individual, antigen-antibody
complexes will form quickly whereas cleavage of the
therapeutic agent will occur slowly, thus resulting in
20 release of the compound at the target site.

In accordance with one embodiment of the invention,
the substrate linkers are modified, for example, by attaching
hydrazine or hydrazide derivative to one end of the linker.
25 The unmodified sites on the linker may or may not be
covalently attached to a therapeutic agent. For instance,
the substrate linkers which are attached to a therapeutic
agent via an ester or amide link, as described in Section 5.3
(see Table II and Table III) are modified by attaching a
30 hydrazide (e.g., phenylhydrazine) to the opposite amino
terminus of the peptide chain. This results in the following
structure (N.B., the symbol * signifies an amide or ester
bond):

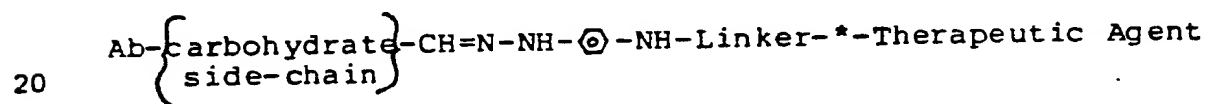


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Although in the structure shown the hydrazine is in the para position, one may alternatively use compounds with the hydrazine moiety in the ortho or meta positions. These hydrazide derivatives of the peptide linkers which are attached to a therapeutic agent via an ester or amide bond are then reacted with an oxidized immunoglobulin, or immunoglobulin fragment containing an oxidized carbohydrate. This results in hydrazone formation and the covalent attachment of the therapeutic agent to the carbohydrate side chain of the immunoglobulin via a linker group which is susceptible to cleavage by complement. If desired, the linker utilized may be resistant to cleavage by either activated complement or serum proteases.

In embodiments in which the linker is designed to be susceptible to cleavage by a serum protease, one resulting structure is schematically represented below (N.B., the symbol * signifies an amide or ester bond):



5.4.6. NON-CLEAVABLE LINKERS OR DIRECT ATTACHMENT

In still other embodiments of the invention, the conjugate may be designed so that the therapeutic agent is delivered to the target but not released. This may be accomplished by attaching a therapeutic agent to an antibody or antibody fragment either directly or via a non-cleavable linker.

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cancers, adenomas, and hyperplasias; certain immunological disorders, including autoimmune diseases, graft-versus-host diseases (e.g., after bone marrow transplantation), immune suppressive diseases, e.g., after kidney or bone marrow transplantation. Treatment of such cellular disorders
5 involving, for example, bone marrow transplantation, may include purging (by killing) undesired cells, e.g., malignant cells or mature T lymphocytes.

Therapeutic applications center generally on
10 treatment of various cellular disorders, including those broadly described above, by administering an effective amount of the antibody-therapeutic agent conjugates of the invention. The properties of the antibody are such that it
15 is immunospecific for and immunoreactive with a particular antigen render it ideally suited for delivery of therapeutic agents to specific cells, tissues, organs or any other site having that particular antigen.

According to this aspect of the invention, the
20 antibody or antibody fragment of the antibody therapeutic agent conjugate functions to deliver the conjugate to the target site.

The choice of antibodies, linkers, and compounds
25 used to make the conjugates depends upon the purpose of delivery. The delivery and release or activation of therapeutic agents at specific target sites may result in selective killing or inhibition of proliferation of tumor cells, cancer cells, fungi, bacteria, parasites, or virus. The
30 targeted delivery of hormones, enzymes, or neurotransmitters to selected sites may also be accomplished. Ultimately the method of the present invention may have an application in gene therapy programs wherein DNA or specific genes may be delivered in vivo or in vitro to target cells that are

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deficient in that particular gene. Additionally, the conjugates may be used to "turn off" or prevent the activation of oncogenes, such as myc, ras and the like.

5 In vivo administration may involve use of
therapeutic agents of antibody therapeutic agent conjugates
in any suitable adjuvant including serum or physiological
saline, with or without another protein, such as human serum
albumin. Dosage of the conjugates may readily be determined
10 by one of ordinary skill, and may differ depending upon the
nature of the cellular disorder and the therapeutic agent
used. Route of administration may be parenteral, with
intravenous administration generally preferred.

15 5.5.1. PHOTORADIATION THERAPY

One type of photoradiation therapy (also referred
to in this context as photoimmunotherapy) which
advantageously uses the antibody-therapeutic agent conjugates
of this invention encompasses the treatment of disorders by
20 combining the phototoxic effects of certain compounds and the
site specific attachment of the antibody to a target site.
The photosensitizer is activated by a light source and its
cytotoxic effect is mediated through the production of
singlet oxygen which results in toxicity to neighboring
25 cells. This effect involves the participation of molecular
oxygen. (For a more complete overview of this topic see
Parrish, 1981, J. Investig. Derm. 77:45-50).

30 The specificity of the photochemical reaction can
be maintained by selecting the proper wavelength and specific
photosensitizer (or chromophore) to be used depending on the
biologic effect desired. It may be possible to attach more
than one photosensitizer for delivery to a target site.
Depending upon the wavelength and effect desired therapeutic
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agents might be activated in a synergistic fashion. The photosensitizer may be activated at the target site with lasers or other light sources via optical fibers or any other appropriate method.

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5.5.2. SUBSTRATE MODIFICATION

In an alternate embodiment of the present invention, substrate activation by the therapeutic agent may be used to mediate formation of singlet oxygen or peroxides and induce cell killing. In this particular embodiment, the therapeutic agent is an enzyme. For example, galactose oxidase will oxidize galactose and some galactose derivatives at the C₆ position. In the course of the oxidation reaction, molecular oxygen is converted into hydrogen peroxide which is toxic to neighboring cells. The enzyme glucose oxidase, a flavoenzyme, may also be used in the embodiment of this invention. This enzyme is highly specific for β-D-glucose and can act as an antibiotic due to peroxide formation. The enzyme may be attached to an antibody molecule either directly or via a non-cleavable linker. An individual is given an effective dosage of this conjugate and is then perfused with substrate. Cell killing is mediated through the formation of peroxides by the methods described above. The toxic effect of peroxides may be amplified by administration of a second enzyme, preferably of human origin to convert the peroxide to a more toxic hypochlorous acid. Examples of suitable enzymes include but are not limited to myeloperoxidase, lactoperoxidase and chloroperoxidase.

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5.6. ADVANTAGES OF ANTIBODY-
THERAPEUTIC AGENT CONJUGATES

According to one embodiment of the present invention, a therapeutic agent may be attached to an antibody directed against a target antigen. The chemical linking methods described herein allow the resulting antibody conjugate to retain the ability to bind antigen and to activate the complement cascade (when the unconjugated antibody or antibody fragment had such ability). As a result, when the conjugate is administered to an individual, the subsequent formation of immune complexes with target antigens in vivo activates the individual's serum complement. If the linker is designed to be susceptible to cleavage by complement, the compound will be cleaved at the target site by one or more of the enzymes of the complement cascade. Since release of the compound occurs after delivery to the target site the efficiency of the target delivery system is greatly improved.

The method of the present invention offers another advantage over other targeting systems. For example, it is known that all cells of a tumor do not each possess the target antigenic determinant. Thus, delivery systems which require internalization into the target cell will effect successful delivery only to those tumor cells that possess the antigenic determinant and that are capable of internalizing the conjugate. Tumor cells that do possess the antigenic determinant or are incapable of this internalization, will escape treatment.

According to the method of the present invention, antibody carrier molecules deliver the therapeutic agent to the target cells. More importantly, however, once attached to the target cell, the method described in the present

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invention allows the release or activation of the active or
activatable therapeutic compound. Release or activation may
be mediated by the individual's activated complement enzymes,
tissue plasminogen activator, urokinase, plasmin or another
enzyme having proteolytic activity, or by activation of a
5 photosensitizer or substrate modification. Once released,
the therapeutic agent is then free to permeate the target
sites, e.g., tumor mass. As a result, the therapeutic agent
will act on tumor cells that do not possess the antigenic
determinant. Additionally, the entire process is not
10 dependent upon internalization of the conjugate.

The following examples will serve to further typify
the nature of the invention without being a limitation on the
scope thereof.

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6. EXAMPLES: SERIES I

The purpose of this series of examples is to
20 demonstrate that the methods for preparing antibody
conjugates described in the present invention do not
adversely affect the antigen binding properties of antibodies
in the way the carbodiimide reaction affects such properties.
To this end, the carbohydrate moieties of a mouse monoclonal
25 IgM, specific for the phosphorylcholine group, were oxidized
and covalently attached to the 1,6-diaminohexyl derivative of
ethylene diamine di- (o-hydroxyphenylacetic acid) [EDDHA] to
form 1,6-diaminohexyl-EDDHA. For comparative purposes, 1,6-
diaminohexyl-EDDHA as well as unmodified EDDHA were attached
30 to identical samples of IgM monoclonal antibody using the
carbodiimide reaction. Under these conditions, the 1,6-
diaminohexyl-EDDHA would couple to available aspartic and
glutamic acid residues, while the unmodified EDDHA would
couple to available lysines.

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The binding properties of these samples were compared with the native antibody in order to evaluate affinity and homogeneity.

5

6.1. OXIDATION OF MOUSE MONOCLONAL IgM

A mouse monoclonal IgM antibody specific for the ligand, phosphorylcholine, was oxidized at a concentration of 2 mg/ml in phosphate buffered saline (PBS, 0.01 M phosphate, 0.15 M sodium chloride), pH 6.0. The antibody-containing solution was cooled in a water-ice bath, and 56.8 µg of sodium metaperiodate was added (40 µl of a 1.42 mg/ml solution; final periodate concentration = 0.26 mM). This reaction mixture was incubated for one hour, after which 2 µl of ethylene glycol was added. This was incubated an additional thirty minutes. The sample was then passed through a Sephadex® G-25 column equilibrated with PBS and the protein fractions pooled.

20

6.2. ATTACHMENT OF LINKER TO EDDHA

EDDHA (1.5 g, 4.2 mmole) and triethylamine (1.2 ml, 8.4 mmole) were mixed with 40 ml of water. This heterogeneous solution was heated to 60°C. and stirred vigorously for 0.5 hours. The solution was dried in vacuo and then was dissolved in 400 ml of dry N,N-dimethylformamide. The solution was then cooled in an ice bath and isobutylchloroformate (0.56 ml, 4.2 mmole) was added. The reaction mixture was stirred with cooling for 0.5 hours. The resulting triethylamine hydrochloride precipitate was removed by filtration and the filtrate containing the mixed carboxycarbonic anhydride of EDDHA was red in color.

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1-amino-6-trifluoroacetamidohexane (0.8 g, 4.1 mmole) was added to the above carboxycarbonic anhydride of EDDHA. The homogeneous solution was stirred at 4°C for 0.5 hours, then was lyophilized to yield an oily product. The oil was washed with an actone/ether (4:1) mixture to yield a crude yellow product. The solid 1-amino-6-trifluoroacetamidohexyl-EDDHA was collected and hydrolyzed with 7% K_2CO_3 and reprecipitated with HCl at pH 4 to yield pure 1,6-diaminohexyl-EDDHA (1.4 g). This compound gives a positive ninhydrin test and thin layer chromatography shows only one spot. In the presence of basic solution of an equal molar quantity of $TbCl_3$, excitation at 295 nm yielded emission at 545 nm, due to formation of the characteristic energy transfer chelate complex between EDDHA and terbium ion.

6.3. PREPARATION OF IgM-LINKER-EDDHA CONJUGATES

The antibody, oxidized by the method of Section 6.1, was incubated with an approximately 270-fold molar excess of 1,6-diaminohexyl-EDDHA, prepared by the method of Section 6.2, for one hour at room temperature. This was followed by addition of solid sodium cyanoborohydride to a final concentration of 10 mM, and further incubation of 4 hours at room temperature. The mixture was then dialyzed at 4°C versus several changes of PBS, and concentrated by ultrafiltration.

6.4. CARBODIIMIDE ATTACHMENT OF LINKER-EDDHA TO IgM

To 263 μ l IgM antibody (1.9 mg/ml) was added 10 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (1 ml of 10 mg/ml solution, pH 5.0) and PBS (pH 5.0) to make up to 2.5 ml. The mixture was incubated two hours at room temperature. Then 275 μ l of 0.1M 1,6-diaminohexyl-EDDHA in 2.5 ml of water

(pH 5.5) was added, and the solution incubated for two hours at room temperature. Ten μ l of 1M ethanolamine was then added and incubated for one hour at room temperature. This was then dialyzed overnight against PBS (pH 7.0).

5

6.5. CARBODIIMIDE ATTACHMENT OF EDDHA TO IgM

To 263 μ l IgM antibody (1.9 mg/ml) was added 10 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (1 ml of 10 mg/ml solution, pH 5.0) and PBS (pH 5.0) to make up to 2.5 ml. The mixture was incubated for two hours at room temperature. To this was added 2.75 ml of 0.01M EDDHA (pH 5.5) and the solution was incubated for two hours at room temperature. Ten μ l 1M of ethanolamine was added and the mixture incubated for one hour at room temperature.

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6.6. EFFECTS OF CARBOHYDRATE-MEDIATED ATTACHMENT TO ANTIBODIES

The affinities of unmodified mouse monoclonal antibody and antibody conjugates prepared according to Sections 6.3, 6.4 and 6.5, all specific for the phosphorylcholine group, were measured by fluorescence quenching according to the methods described by Rodwell and Karush, 1983, J. Immunol. 130:313-316, incorporated herein by reference.

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The Sips plots presenting data for antibody carbodiimide conjugates and antibody conjugates of the invention are shown in FIG. 2. The binding measurements clearly demonstrate the retention of specificity, affinity, and homogeneity for the sample modified via the carbohydrate attachment methods of the invention, (Δ — Δ), when compared to

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1-amino-6-trifluoroacetamidohexane (0.8 g, 4.1 mmole) was added to the above carboxycarbonic anhydride of EDDHA. The homogeneous solution was stirred at 4°C for 0.5 hours, then was lyophilized to yield an oily product. The oil was washed with an actone/ether (4:1) mixture to yield a crude yellow product. The solid 1-amino-6-trifluoroacetamidohexyl-EDDHA was collected and hydrolyzed with 7% K_2CO_3 and reprecipitated with HCl at pH 4 to yield pure 1,6-diaminohexyl-EDDHA (1.4 g). This compound gives a positive ninhydrin test and thin layer chromatography shows only one spot. In the presence of basic solution of an equal molar quantity of $TbCl_3$, excitation at 295 nm yielded emission at 545 nm, due to formation of the characteristic energy transfer chelate complex between EDDHA and terbium ion.

6.3. PREPARATION OF IgM-LINKER-EDDHA CONJUGATES

The antibody, oxidized by the method of Section 6.1, was incubated with an approximately 270-fold molar excess of 1,6-diaminohexyl-EDDHA, prepared by the method of Section 6.2, for one hour at room temperature. This was followed by addition of solid sodium cyanoborohydride to a final concentration of 10 mM, and further incubation of 4 hours at room temperature. The mixture was then dialyzed at 4°C versus several changes of PBS, and concentrated by ultrafiltration.

6.4. CARBODIIMIDE ATTACHMENT OF LINKER-EDDHA TO IgM

To 263 μ l IgM antibody (1.9 mg/ml) was added 10 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (1 ml of 10 mg/ml solution, pH 5.0) and PBS (pH 5.0) to make up to 2.5 ml. The mixture was incubated two hours at room temperature. Then 275 μ l of 0.1M 1,6-diaminohexyl-EDDHA in 2.5 ml of water

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7. EXAMPLES: SERIES II

The following examples illustrate methods for the specific attachment of an antibody molecule to a compound of interest via a linker.

While the compound attached in these experiments is not considered a therapeutic agent, its use illustrates specific covalent attachment to the oxidized carbohydrate moiety of an antibody via a linker. By analogous mechanisms, a therapeutic agent may be attached to prepare an antibody-therapeutic agent conjugate.

7.1. OXIDATION OF THE CARBOHYDRATE MOIETY OF THE ANTIBODY MOLECULE

The antibody molecule used in this example was a monoclonal IgM (designated no. 171) specific for antigenic determinants on sheep red blood cells. To prepare the monoclonal antibody, Lewis rats were immunized with a single injection of sheep red blood cells. Three days later, spleen cells from the immunized rats were harvested and fused with the myeloma line SP2/O Ag14 according to the method of McKearn *et al.*, 1979, *Immunol. Rev.* 47:91-115. Cloned cells were then grown and the resulting monoclonal antibody was purified as described by Kliman and McKearn, 1981, *J. Immunol. Meth.* 42:1-9.

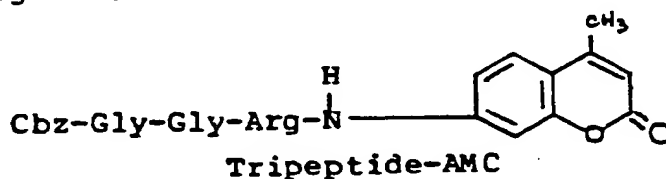
Oxidation of the antibody carbohydrate moiety was accomplished by reacting the antibody with galactose oxidase by a modification of the method of Cooper *et al.*, *supra*. To this end, 3.8 mg of no. 171 monoclonal antibody was added to 1 ml of buffer consisting of 0.135 M NaCl, 0.015 Tris-HCl (pH 7.0), 0.5 mM MgCl₂, and 0.15 mM CaCl₂. Subsequently, a 0.1 ml aliquot of a solution of galactose oxidase (Worthington

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Biochemical Co., Freehold, NJ) at a concentration of 52 units of enzyme/ml of the same buffer was added to the antibody solution. Finally, 43 μ g of catalase (Worthington Biochemical Co., Freehold, NJ) dissolved in an additional 0.1 ml of the same buffer was added to the reaction mixture (the catalase was added to degrade hydrogen peroxide that is generated during the oxidation reaction). The reaction mixture was incubated for 48 hours at room temperature, then stored at 4°C. FIG. 3 represents the excitation spectra for unoxidized (a) and oxidized (b) antibodies.

7.2. PREPARATION OF THE TRIPEPTIDE-AMC FOR ATTACHMENT TO THE ANTIBODY MOLECULE

For the purposes of the present example, a synthetic fluorogenic compound was utilized as the conjugate partner. The properties of this synthetic compound are such that the bound and free states of the fluorogenic compound are spectrofluorometrically distinguishable. The synthetic fluorogenic compound used was obtained from Serva Fine Biochemicals, Inc., Garden City Park, LI, NY (Catalog #51474). This compound consists of a tripeptide (Gly-Gly-Arg) attached via an amide linkage to the fluorescent compound 7-amino-4-methyl coumarin (AMC); the amino group of glycine is blocked by carbobenzoxy chloride (Cbz). The structure of this compound (hereinafter Tripeptide-AMC or Gly-Gly₇Arg-AMC) is shown below:



The excitation and emission maxima of free AMC (345 nm and 445 nm, respectively) differ from those for AMC bound

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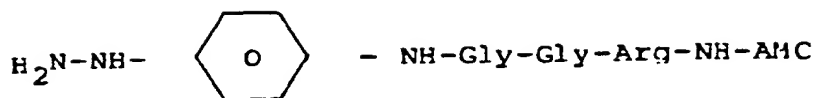
to the tripeptide (325 nm and 395 nm, respectively). This affords a means for distinguishing between the bound and free forms of the AMC molecule using a fluorometric assay.

Excitation and emission wavelengths of 383 nm and 455 nm may be used for optimum differences for assay purposes; at these
5 wavelengths, free AMC retains 20% of its maximal fluorescence but possesses a relative fluorescence 500-fold greater than an equimolar amount of bound AMC (Zimmerman et al., 1978, Proc. Natl. Acad. Sci., U.S.A. 75(2):750-753).

10 A hydrazine derivative of the Tripeptide-AMC compound was prepared. Aldehyde groups of the oxidized carbohydrate side chain of the antibody molecule were then reacted with the hydrazine derivative to form a hydrazone.

15 In order to attach a hydrazine derivative (e.g., 4-fluorophenylhydrazine), the Tripeptide-AMC was first deblocked at the glycine amino terminus by removal of the Cbz group. This was accomplished by dissolving the Tripeptide-AMC in trifluoroacetic acid (Sigma, St. Louis, MO), and
20 bubbling HBr gas (Matheson, East Rutherford, NJ) through the solution for 45 minutes. The product, H₂N-Gly-Gly-Arg-NH-AMC, was precipitated by the addition of cold diethyl ether (Baker Chemical Co., Phillipsburgh, NJ), and dissolved in absolute ethanol (Publicker Industries Co., Linfield, PA).
25 An equimolar amount of 4-fluorophenylhydrazine (Aldrich Chemical Co., Milwaukee, WI) in absolute ethanol was added with mixing. After incubation in the dark at room temperature for 2 hours, the reaction mixture was stored in the dark at 4°C. The resulting product (Phenylhydrazine-Tri-
30 peptide-AMC) has the structure:

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This compound was shown to be positive for fluorescence by exciting with ultraviolet light, and positive for the presence of a hydrazine group. The hydrazine linked to the tripeptide was detected by thin layer chromatography (TLC) using a spray of a 0.1% trinitrobenzene sulfonic acid aqueous solution for the colorimetric determination of a hydrazine (a pinkish or orange-brown color indicates the presence of hydrazine). The results of TLC demonstrated the presence of a hydrazine group at the migratory band of the Tripeptide-AMC.

The absorption and emission spectra for the Phenylhydrazine-Tripeptide-AMC compound as shown in FIG. 4 reveal a similarity to the Tripeptide-AMC spectra, but a shift in excitation and emission maxima consistent with the covalent modification of the Phenylhydrazine-Tripeptide-AMC. The maxima for excitation and emission of the Phenylhydrazine-Tripeptide-AMC compound are 345 nm and 385 nm, respectively. The product was precipitated from solution with cold diethyl ether, washed, and dissolved in dimethylsulfoxide (Baker Chemical Co., Phillipsburgh, NJ).

7.3. ATTACHMENT OF PHENYLHYDRAZINE-TRIPETIDE-AMC TO THE OXIDIZED CARBO-HYDRATE MOIETY OF THE ANTIBODY MOLECULE

The oxidized monoclonal antibody preparation described in Section 7.1, supra, was adjusted to pH 5.1 by the addition of a small amount of 0.1 M acetate buffer (pH 5.0). An estimated 10-fold excess of Phenylhydrazine-Tripeptide-AMC (prepared in Section 7.2) was added to the antibody solution, which was then incubated at 37°C in the dark, overnight (approximately 14 hours). The reaction mixture was then chromatographed on a Sephadex® G-25 column

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(Pharmacia Fine Chemicals, Piscataway, NJ) in order to remove any unreacted Phenylhydrazine-Tripeptide-AMC.

Spectrofluorometric analysis of the protein fractions confirmed the presence of the Phenylhydrazine-Tripeptide-AMC covalently attached to the antibody (Antibody-Phenylhydrazine-Tripeptide-AMC). The excitation and emission maxima for the conjugate are 325 nm and 385 nm, respectively (FIG. 5). The large peak at 285 nm in the excitation spectrum of the conjugate may be explained by tryptophan absorption with residual fluorescence at 385 nm and may also be the result of resonance energy transfer from the amino acid tryptophan of the antibody molecule to AMC.

8. EXAMPLES: SERIES III

The following examples illustrate specific release of the compound from the antibody conjugate prepared by the methods of Section 7. These antibody conjugates retain the ability to fix complement as revealed by a hemolytic complement fixation assay. Furthermore, the specific release of the compound from the antibody conjugate, at the antigenic cell surface, via enzymatic cleavage by the complement system is demonstrated by a non-hemolytic assay.

In the following examples the compound is fluorogenic. Thus, the complement mediated release of the fluorescent compound may be detected by an assay capable of differentiating between the bound and free forms of the fluorescent molecule.

While the compound released in this example is not considered a therapeutic agent, its use illustrates enzymatic cleavage of a linker by the complement system or a serum enzyme having proteolytic activity. By analogous cleavage

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mechanisms, a therapeutic agent may be released from an antibody-therapeutic agent conjugate.

5 The materials and procedures of Section 7.1 were used as described to oxidize the carbohydrate moieties of monoclonal antibodies (No. 171).

10 In the presence of sheep red blood cells and serum complement, these monoclonal antibodies (No. 171) activate the complement enzyme cascade (a result of antigen-antibody binding). Complement fixation causes lysis of the sheep red blood cells which results in the release of hemoglobin. The released hemoglobin may be detected spectrophotometrically, thus providing an assay for complement fixation.

15 The Tripeptide-AMC was prepared as described in Section 7.2. The properties of the fluorogenic compound (AMC) are such that the bound and free states of the fluorogenic compound are spectrofluorometrically distinguishable. This provides a definitive assay for
20 measuring the complement fixation ability of the antibody conjugate. More importantly, it provides a means for quantitating the subsequent complement-mediated release of the compound.

25 The specific covalent attachment of phenylhydrazine-tripeptide-AMC to the oxidized carbohydrate moieties of the antibodies was performed as described in Section 7.3.

30

8.1. COMPLEMENT FIXATION ASSAYS

35 Two types of complement fixation assays were utilized, hemolytic and fluorometric. These assays

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determined whether the Antibody-Phenylhydrazine-Tripeptide-AMC conjugate retained complement fixation ability, and whether AMC was cleaved by complement.

5 8.1.1. PREPARATION OF HUMAN COMPLEMENT

10 A 10 ml sample of freshly drawn human whole blood was clotted on ice for 17 hours. The clot was removed by centrifugation, and the resulting human serum was frozen in 0.5 ml aliquots. Human complement was shown to be active in these samples by the hemolytic assay described in Section 8.1.2.

15 8.1.2. HEMOLYTIC ASSAY FOR COMPLEMENT FIXATION

20 A 200 μ l aliquot of a suspension of sheep red blood cells (Gibco Diagnostics, Madison, WI) at an approximate concentration of 2×10^8 cells/ml were mixed with 20 μ l of the Antibody-Phenylhydrazine-Tripeptide-AMC conjugate mixture prepared in Section 7.3 (approximately 2 μ g of protein). After 15 minutes of mixing and incubating at 37°C, 100 μ l. of the human serum complement (prepared in Section 8.1.1.) was added to the mixture. After 30 minutes to 1 hour of incubation at 37°C, the mixture was centrifuged to pellet the cells. The extent of the complement-mediated cell lysis was determined by spectrophotometrically measuring hemoglobin released into the supernatant (412 nm).

30 The results of this assay demonstrated complete hemolysis and essentially 100% binding of antibody to cell surface. For example, addition of distilled water to a pellet formed by centrifuging 200 μ l of the sheep red blood cell suspension completely lyses the cells, and releases hemoglobin. A 1:20 dilution of the supernatant of sheep red

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blood cells which were completely lysed in distilled water had an O.D.₄₁₂ of 0.646. An identical dilution of sheep red blood cells which were lysed by the addition of conjugate and complement had an O.D.₄₁₂ of 0.672. Thus the conjugate retained the ability to bind antigen and to fix complement.

5

8.1.3. NON-HEMOLYTIC ASSAY FOR COMPLEMENT MEDIATED RELEASE OF AMC

Conditions for the non-hemolytic assay were identical to those above except that glutaraldehyde-fixed sheep red blood cells (Sigma, St. Louis, MO) were used in place of normal sheep red blood cells. Glutaraldehyde fixed cells do not lyse in the presence of antibody and complement and, therefore, no hemoglobin is released. Instead, a fluorometric assay is used to demonstrate the release of the AMC. A non-hemolytic system is necessary for use in the fluorometric assay, because the presence of hemoglobin interferes with fluorescence measurements in this system. Prior to use in the assay, these fixed red blood cells were shown to bind both the unmodified antibody and the Antibody-Phenylhydrazine-Tripeptide-AMC which was prepared in Section 7.3.

The non-hemolytic assay was used to show the specific complement-mediated release of the AMC from the antibody conjugate. Similarly to the hemolytic assay, 200 μ l of the glutaraldehyde-fixed sheep red blood cells, at an approximate concentration of 2×10^8 cells/ml, was incubated with the Antibody-Phenylhydrazide-Tripeptide-AMC conjugate at 37°C for 15 minutes.

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After centrifuging and resuspension in buffer, 50 μ l of the human complement preparation (Section 8.1.1) was added, and the fluorescence at 460 nm monitored, with

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excitation at 380 nm (Caporale et al., 1981, J. Immunol. 128:1963-65.) as a function of time. As controls, the conjugate was incubated with sheep red blood cells alone; in the presence of rat red blood cells and human complement (the monoclonal antibody used does not bind to rat red blood cells); and in the absence of both sheep red blood cells and complement (the monoclonal antibody used does not bind to rat red blood cells). FIG. 6 shows the results of these experiments. A comparison of curve (a) which represents the conjugate incubated with glutaraldehyde-fixed sheep red blood cells and human complement to the control curved labeled (b), (c) and (d) clearly demonstrates the release of free AMC in the sample containing the specific antibody target and human complement. Thus, curve (b) which represents the conjugate incubated with glutaraldehyde-fixed rat red blood cells and human complement, curve (c) which represents the conjugate incubated with glutaraldehyde-fixed sheep red blood cells, and curve (d) which represents the conjugate alone demonstrate no release of AMC.

9. EXAMPLES: SERIES IV

According to one embodiment of the present invention a therapeutic agent may be attached to an antibody or antibody fragment via a linker that is susceptible to cleavage by complement or other proteolytic enzymes. Such antibody-conjugates are particularly useful for therapeutic applications where it is desired to release the therapeutic agent at the target site in the body. As detailed in Section 5, the antibody-therapeutic agent conjugates may be prepared either by attaching a therapeutic agent to an antibody-linker intermediate, or by attaching an antibody to a linker-therapeutic agent intermediate.

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According to another method of the present invention, for attachment to reduce antibodies or antibody fragments, the linkers (e.g., Gly-Gly-Arg) must be modified to contain a reactive group capable of reaction with the sulfhydryl group of a reduced antibody or Fab' fragment.

5 Such reactive groups include, but are not limited to: reactive haloalkyl groups (including, for example, haloacetyl groups), p-mercuribenzoate groups and groups capable of Michael-type addition reactions (including, for example, maleimides and groups of the type described by Mitra and

10 Lawton, 1979, J. Amer. Chem. Soc. 101: 3097-3110). By the term "haloalkyl" is meant any alkyl group of one to three carbon atoms substituted with bromine, iodine or chlorine.

In the following examples (Series IV and V) either

15 (AMC) or the amino acid tyrosine, covalently attached to a linker, e.g., Gly-Gly-Arg, was released via enzymatic cleavage. While the compounds (AMC and tyrosine) released by the cleavage of the linker in these examples are not considered therapeutic agents, their use illustrates

20 enzymatic cleavage by serum enzymes such as trypsin, urokinase and plasmin and tissue plasminogen activator. By analogous mechanisms, a therapeutic agent may be cleaved by such enzymes from the linker-therapeutic agent intermediates of the present invention.

25 ; In Series IV, the AMC or tyrosine was enzymatically cleaved from a free linker. In Series V, the AMC or tyrosine was enzymatically cleaved from a free antibody-linker conjugate and from an antibody-linker conjugate bound to a

30 target cell. Thus, comparison of results from the two series of experiments illustrates the effect of the leaving group (AMC or tyrosine) on the rate of cleavage, as well as the effect of the environment (i.e., free linker versus free

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antibody-linker conjugate versus antibody-linker conjugate bound to a target cell).

5 9.1. CLEAVAGE OF THE TRIPEPTIDE-AMC BY TRYPSIN AND UROKINASE

For the purposes of this experiment, a synthetic fluorogenic compound Gly-Gly-Arg-AMC obtained from Serva Fine Biochemicals, Inc., Garden City Park, NY was utilized.

The rates of cleavage for the peptide linker were measured by fluorescence quenching using a Perkin Elmer 650-10S fluorescence spectrometer (Perkin-Elmer Corporation, Norwalk, CT). The excitation and emission wavelengths were 380 nm and 460 nm, respectively and the temperature was maintained at 25°C with a Lauda k-2/R circulating water bath (Brinkmann Instruments, Westbury, NY).

20 The initial concentrations of Gly-Gly-Arg-AMC were determined by optical density, $\sum_{325}^{1M} = 16,000$. The solutions were adjusted to 1 μ M concentrations. One ml of the 1 μ M solution in PBS, pH 7.4 was placed in a cuvette. The appropriate amounts of enzyme (1 μ g/ml trypsin, or 10 μ g/ml urokinase) was then added at room temperature with stirring using a motor-driven syringe and the reaction kinetics were followed for several minutes. The fluorescence intensity of a known concentration of free AMC was established and used as a baseline to determine the change in units per nm/minute of free AMC in this fluorescence assay system. The results of the assay are described in Table IV.

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TABLE IV

Section ¹	Substrate	Released Group Substrate Concentration	Cleavage Rates nM/min.	
			Trypsin ² 1 µg/ml	Urokinase 10 µg/ml
9.1	Gly-Gly-Arg-AMC	1 µM	35	16
9.1	Gly-Gly-Arg-AMC	1 µM	75	60
9.1	Gly-Gly-Arg-AMC	1 µM	170	78
9.1	Dextran-Gly-Gly-Arg-AMC	1 µM	8.4	.9
9.1	Gly-Gly-Arg-AMC	100 µM	20,000	not determined
9.3	Gly-Gly-Arg-Tyr*	0.1 µM	4	.01
9.4	Pro-Gly-Arg-Val- Val-Gly-Tyr*	0.1 µM	3	.01

1 Section = section in which experiment is described.

2 100 µg/ml Trypsin was used in the experiment in which the Gly-Gly-Arg-AMC substrate concentration was 100 µM.

In another experiment, the Gly-Gly-Arg-AMC concentration was 100 µM and the trypsin used at 100 µg/ml. The results of this assay are also described in Table IV.

In another embodiment of the present invention, the Gly-Gly-Arg-AMC was conjugated to oxidized dextran which can serve as a spacer as described in Section 5.4.3 (10,000 molecular weight) (Pharmacia Fine Chemicals, Piscataway, NJ) by the methods of the present invention using a molar ratio of Gly-Gly-Arg-AMC to dextran of 58:1. Dextran-Gly-Gly-Arg-AMC was separated with a 6.5 ml Sephadex® G-25 column (Pharmacia Fine Chemicals, Piscataway, NJ).

Rates of enzymatic cleavage of the dextran-Gly-Gly-Arg-AMC, using the methods of this section, are also summarized in Table IV.

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Peptide sequences that are analogs of complement components were cleaved by trypsin (0.1 $\mu\text{g/ml}$), urokinase (0.1 $\mu\text{g/ml}$), plasmin (1 $\mu\text{g/ml}$) and purified complement component (40 $\mu\text{g/ml}$). The data in Table V compare the
5 cleavage rates (nM/minute) of these sequences. The fluorescence assay described in Section 8 for cleavage of Gly-Gly-Arg-AMC was used here. Complement components were purified and assayed by the method of Tack and Prahl (Biochem., 1976, 15:4513-4521).

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35 30 25 20 15 10 5

TABLE V

Cleavage of Tripeptide-AMC Derivatives
by Urokinase, Trypsin, Plasmin and
Purified Components C4,2 and Cls

Cleavage Rates, nM/min					
PEPTIDE (5 μ M)	Urokinase (0.1 μ g/ml)	Trypsin (0.1 μ g/ml)	Plasmin (1 μ g/ml)	ClS, C4,2 (40 μ g/ml)	ClS (10 μ g/ml)
Z-Gly-Gly-Arg-AMC	27	450	0.005	0.9-1.2	0.9-1.2
Glu-Gly-Arg-AMC	35	140	--	--	--
Boc-Val-Gly-Arg-AMC	2.8	720	--	--	--
Boc-Leu-Gly-Arg-AMC	3.7	750	--	--	--
Boc-Val-Gly-Arg-AMC	2.8	720	--	--	--
Boc-Phe-Ser-Arg-AMC	0.044	17	--	--	--
Boc-Val-Pro-Arg-AMC	0	3.8	--	--	--
Boc-Glu-Arg-Arg-AMC	0	3.7	--	--	--
Glu-Lys-Lys-AMC	0	0.62	--	--	--
Pro-Phe-Arg-AMC	0	0	--	--	--

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9.3. CLEAVAGE OF GLY-GLY-ARG-TYR* BY UROKINASE AND TRYPSIN

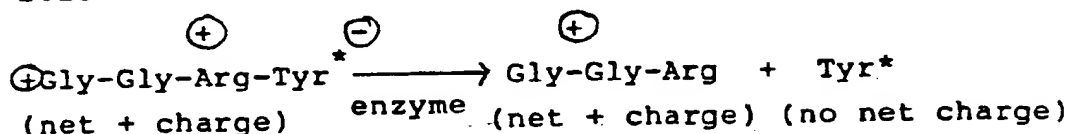
For the purposes of the present example, the tetra-peptide Glycine-Glycine Arginine-Tyrosine was radiolabeled at the amino acid residue tyrosine with 125 Iodine (125 I) (Greenwood et al. 1963, Biochem. J. 88: 114-120). This radiolabeled compound is hereinafter referred to as Gly-Gly-Arg-Tyr* (wherein the * denotes 125 I). The properties of this radiolabeled peptide are such that the bound and free states can be readily assayed for the release of the 125 I labeled tyrosine residue after cleavage with the appropriate amount of enzyme (1 μ g/ml trypsin or 10 μ g/ml urokinase).

The peptide Gly-Gly-Arg-Tyr* was assayed for the release of the 125 I labeled tyrosine residue by agarose gel electrophoresis or thin-layer chromatography (TLC, Sono and Asakura, 1974, Biochem. 13:4386-4394). Briefly, appropriate amounts of enzyme were added to a reaction tube containing 0.1 μ M Gly-Gly-Arg-Tyr* in PBS, pH 7.4. The mixture was incubated at 37°C for 30 minutes. Three μ l of this reaction mixture were then applied to an agarose gel (1%) and run at high voltage (120 V/cm) in Barbitol Buffer, 0.025 M sodium diethylbarbituate HCl buffer, pH 8.6. Three μ l of bromophenol blue solution was used as a marker to determine the end of the run. The agarose gel was then cut into sections and counted in a LKB 1271 gamma counter (LKB Instruments, Gaithersburg, MD) to determine if the Tyr* remained at the origin or migrated through the gel. Alternatively, samples were separated and identified by TLC as described supra. Samples were spotted on TLC plates after incubation with the enzyme and run in 4:1:1 BAW solvent (butanol:acetic acid:water) until the solvent front was one inch from the top of the plate. The plate was dried and divided into ten sections which were counted in a gamma counter. The 125 I

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labeled tyrosine residue will migrate further than the unlabeled peptide in this system.

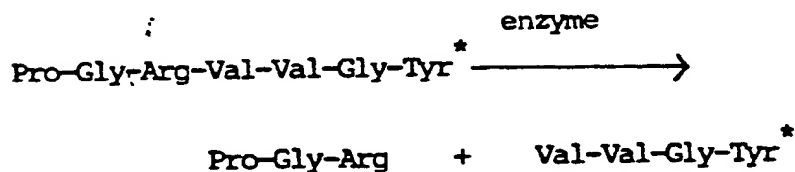
In both systems the peptide is cleaved as described below:



The rates of cleavage for trypsin and urokinase are summarized in Table IV.

9.4. CLEAVAGE OF A PLASMINOGEN ANALOG BY TRYPSIN AND UROKINASE

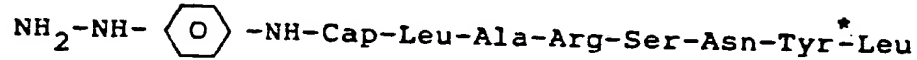
In the following experiments, an ^{125}I radiolabeled plasminogen analog Pro-Gly-Arg-Val-Val-Gly-Tyr^{*} was synthesized by standard peptide solid phase synthetic methods (Barany and Merrifield, IN The Peptides, Vol. 2, E. Gross and J. Meinhofer, eds., Academic Press, NY, 1980, pp 1-180). It was cleaved by the enzymes trypsin (1 $\mu\text{g/ml}$) and urokinase (10 $\mu\text{g/ml}$), as described above and assayed by TLC in the BAW solvent system described supra. The peptide is cleaved as described below:



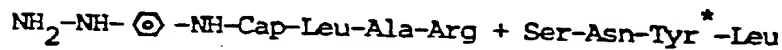
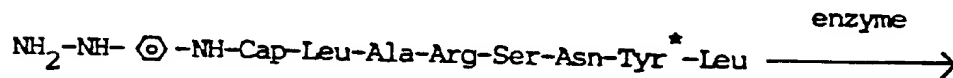
In these assays the radiolabeled Tyr^{*} residue of the peptide migrated farther than the unlabeled portion. The rates of cleavage are summarized in Table IV.

9.5. CLEAVAGE OF A PURIFIED COMPLEMENT COMPONENT ANALOG

In another experiment, an ^{125}I radiolabeled C3 complement component analog



covalently attached to phenylhydrazine aminocaproic acid (NH-Cap) prepared by solid phase synthesis (Barany and Merrifield, supra) was cleaved by purified complement component C1s (10 $\mu\text{g/ml}$) and C1N, C4,2 (40 $\mu\text{g/ml}$). Complement components were purified by the method of Tack and Prahl, 1976, Biochem. 15:4513-4521. The peptide is cleaved as follows:



In these assays the radiolabeled Tyr^{*} residue of the peptide migrated farther than the unlabeled portion. The rates of cleavage are summarized in Table VI.

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TABLE VI
ENZYMATIC CLEAVAGE

5	Substrate	Released Group Substrate Concentration	Cleavage Rates nM/min.	
			Cls 10 µg/ml	Cls, C4,2 40 µg/ml
	NH ₂ -NH-⊖-NH-Cap-Leu-Ala-	5 µM	.01	.01
10	Arg-Ser-Asn-Tyr [*] -Leu			

10. EXAMPLES: SERIES V

15 Two specific monoclonal antibodies were utilized in the following experiments (NS 4.1 - Mouse IgM against sheep red blood cells and LL 1151-Mouse IgG against sheep red blood cells).

20 10.1. FORMATION OF ANTIBODY-GLY-GLY-ARG-TYR^{*} CONJUGATES

25 Radiolabeled antibody-Gly-Gly-Arg-Tyr^{*} conjugates were prepared according to one method of the present invention as described below. The carbohydrate moiety of the antibody (LL 1151) was oxidized by reacting approximately 1 mg/ml of antibody in phosphate buffered saline (PBS) with 110 µl of 100 mM sodium metaperiodate (NaIO₄) at pH 6 (to give a final
30 concentration of 10 mM (NaIO₄) for 1 hour on ice in the dark.

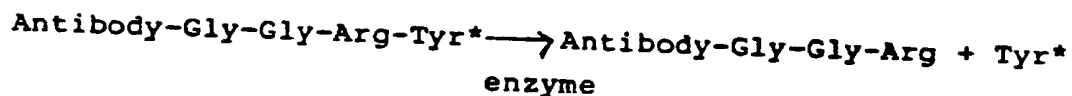
35 Gly-Gly-Arg-Tyr^{*}, prepared by the method of Section 9.3, was coupled to normal human IgG or LL 1151

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or NS 4.1 antibody oxidized by the method of Section 12.1 infra, by incubating the antibody at a 300-fold molar excess of Gly-Gly-Arg-Tyr* in the 0.1 M phosphate buffer, pH 6.0. To reduce the imine, sodium cyanoborohydride (NaCNBH₃) was added to a final concentration of 10 mM, and the reaction mixture was maintained at room temperature for 2 hours. Unreacted Gly-Gly-Arg-Tyr* was separated from the antibody conjugate by gel filtration. The sample was passed through a 5 ml Sephadex® G-50 column (Pharmacia Fine Chemicals, Piscataway, NJ), which had been pre-coated with 1 ml of 10% BSA and run in PBS, pH 7.4. The protein fraction were pooled.

10.2. CLEAVAGE OF ANTIBODY-GLY-GLY-ARG-TYR* CONJUGATES BY TRYPSIN AND UROKINASE

The antibody-Gly-Gly-Arg-Tyr* conjugates prepared by the method of Section 10.1 were assayed for the release of the ¹²⁵I tyrosine residue by gel filtration after cleavage of the linker. The conjugate was cleaved by the enzyme trypsin (100 µg/ml or 10 µg/ml) or urokinase (100 µg/ml) as described in section 9.2, and the mixture was chromatographed on a Sephadex® G-50 column. In this system, the cleaved Tyr* was retarded by the column. Therefore cleavage was determined by the loss of radioactivity in the void volume peak after gel filtration on the column. The antibody-peptide was cleaved as follows:



The results of this experiment are summarized in Table VII.

TABLE VII

ENZYMATIC CLEAVAGE OF ANTIBODY CONJUGATES

Section	Substrate	% cleaved (based on loss of ¹²⁵ Tyr)			
		Trypsin		Urokinase	
		100 μg/ml	10 μg/ml	100 μg/ml	10 μg/ml
10.2	NS 4.1-Gly-Gly-Arg-Tyr*	33	27	3	
10.2	LL 1151-Gly-Gly-Arg-Tyr*	33	18	1	
10.3	GSRBC-NS 4.1-Gly-Gly-Arg-Tyr*		28		6
10.3	GSRBC-LL 1151-Gly-Gly-Arg-Tyr*		19		-

¹ Section = section in which experiment is described.

10.3. ATTACHMENT OF THE ANTIBODY-GLY-GLY-ARG-TYR* CONJUGATES TO CELLS AND ENZYMATIC CLEAVAGE

This experiment illustrates cleavage of Tyr* from antibody-Gly-Gly-Arg-Tyr* conjugates, wherein the antibody conjugate was attached to an antigenic determinant of a cell.

Twenty ml of glutaraldehyde-fixed sheep red blood cells (GSRBC, 1×10^9 cells/ml) were washed in Buffer I (0.15 M NaCl, 0.05 M Tris, 0.1 mg/ml BSA, pH 8.0) and resuspended in 2 ml of Buffer I. The cells were incubated with LS 1151-Gly-Gly-Arg-Tyr* (or NS 4.1 Gly-Gly-Arg-Tyr*) prepared by the method of Section 10.1 for 30 minutes at 0°C, then for 30 minutes at 37°C, washed several times with Buffer I and finally resuspended in 10 ml Buffer I. Either of the enzymes trypsin (10 μg/ml) or urokinase (10 μg/ml) was added to 1 ml aliquots of cells and incubated for 0, 15, 60, and 180 minutes at 37°C. Controls were treated in a similar manner but without the addition of enzyme. After incubation at 37°C

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5 the cells were washed with buffer, resuspended in 1 N NaOH, and counted in a LKB 1271 gamma counter (LKB Instruments, Gaithersburg, MD). The percent of loss of counts per minute (CPM) compared to the control was calculated to be the amount of Tyr* cleaved.

The results are summarized in Table VII.

10 10.4. FORMATION AND CLEAVAGE OF CELL ANTIBODY
GLY-GLY-ARG-AMC

This experiment illustrates the cleavage of AMC from an antibody-Gly-Gly-Arg-AMC conjugate attached to a target cell.

15 Two ml of glutaraldehyde fixed sheep red blood cells (GSRBC, 1×10^9 cells/ml) were washed in EDTA (ethylenediaminetetraacetic acid) - VBS gel buffer. The pellet was incubated with an LL 1151 Gly-Gly-Arg-AMC conjugate, prepared using the same methods described in
20 Section 12.1 infra and incubated for 30 minutes at 37°C, and then for 30 minutes at 0°C. This mixture was washed with EDTA-VBS gel buffer and resuspended in 2.2 ml PBS, pH 7.4. The mixture was separated into two portions. Trypsin
25 (1 µg/ml) or urokinase (1 µg/ml) was then added to one portion of the resuspended material. The other portion was the control. The conjugate was then incubated at 37°C. At various times during the incubation period, the mixture was centrifuged and the fluorescence of the supernatant was
30 measured using a Perkin Elmer 650-10S fluorescence spectrophotometer as described in Section 9.1. The fluorescence of the known concentration of bound AMC (control) was determined and the change in nM/minute after cleavage was calculated.

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The results are summarized in Table VIII.

TABLE VIII

CLEAVAGE OF AMC FROM ANTIBODY
CONJUGATE ATTACHED TO TARGET CELL

	Substrate	Trypsin	Cleavage Rate (nM/min.)
			1 µg/ml Enzyme Urokinase
	GSRBC-LL 1151- Gly-Gly-Arg-AMC	0.48	0.14

11. EXAMPLES: PREPARATION OF PORPHYRIN
DERIVATIVES

The following examples illustrate the novel derivatives of porphyrin which can be used as photo-activatable cytolytic agents (or photosensitizers) for in vivo therapy when specifically attached to an antibody molecule.

11.1. PREPARATION OF DEUTEROPORPHYRIN
DIHYDRAZIDE

Commercially available deuteroporphyrin dimethyl ester (98%, 0.05 g, 0.093 mmole), obtained from Aldrich Chemicals, Milwaukee, WI, was suspended in 10 ml dry methanol and 10 µl anhydrous hydrazine (9.0 mg, 0.28 mmole) was added. The solution was refluxed for approximately 4 hours and then evaporated to produce a deep red solid.

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11.2. PREPARATION OF PROTOPORPHYRIN
DIHYDRAZIDE

5 Commercially available protoporphyrin dimethyl
ester (345 mg, 6.0 mmole) obtained from Aldrich Chemicals,
Milwaukee, WI, was suspended in 30 ml dry methanol.
Anhydrous hydrazine (48.0 mg, 1.5 mmole, 47.4 μ l) was added
and the solution refluxed for 24 hours. Evaporation gave a
deep red solid which was only slightly soluble in many
10 organic solvents.

11.3. PREPARATION OF HEMATOPORPHYRIN DIMETHYL
ESTER

15 Commercially available hematoporphyrin (3.0 g, 5
mmole) was obtained from Aldrich Chemicals, Milwaukee, WI,
suspended in 50 ml dry methanol in a 250 ml erlenmeyer flask
equipped with a magnetic stirrer and cooled to 0°C in an ice
bath. A solution of ethereal diazomethane (approximately 17
20 mmoles in about 125 ml ether) was added slowly over a period
of 25 minutes with constant stirring. The mixture was
stirred at 0°C for one hour and then at room temperature
overnight in the dark. Evaporation of the solution produced
a deep red solid which was separated by the technique of
25 thin-layer chromatography (Sono and Asakura, 1974, Biochem.
J. 13:4356-4394) using a developing solvent composed of 5%
methanol and 95% chloroform. Several components were
identified, including a major component which gave a large
pink spot with an R_f value of 0.14. The dimethyl ester could
30 be converted to the corresponding dihydrazide by standard
treatment with methanolic hydrazine.

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11.4. PREPARATION OF HEMATOPORPHYRIN DIHYDRAZIDE

Hematoporphyrin free base (0.5 g, 8.35×10^{-4} mole, Sigma Chemical Co., St. Louis, MO) was dissolved in 200 ml of dry N,N-dimethylformamide and 0.24 ml of triethylamine was added and stirred under nitrogen gas for 20 minutes at room temperature. This homogeneous solution was cooled in an ice bath and 0.218 ml isobutylchloroformate (1.67×10^{-3} mole) was added. After stirring for one-half hour, 0.132 ml (4.16×10^{-3} mole) anhydrous hydrazine was added. The solution was stirred in the dark at 4°C for one hour and for one hour at room temperature.

The solution was dried with a Rotary Evaporator (Rotavap, Buchi, Brinkmann Instruments, Westbury, NY) to give a deep red residue. Distilled water was added to the residue and the solution was adjusted to pH 12 with 1N NaOH to yield more of the precipitate. The solid product was removed by filtration, washed with distilled water and dried in a vacuum dryer to give a deep red crystals.

11.5. PREPARATION OF FICOLL-HYDRAZIDE-HEMATOPORPHYRIN

Ficoll 70 was obtained from Pharmacia Fine Chemicals, Inc., (Piscataway, NJ). The carboxymethyl derivative of Ficoll 70 (CM-Ficoll) was prepared by a modification of the method of Inman (1975, J. Immunol. 114: 704-709). Two grams of CM-Ficoll were dissolved in 100 ml of water and 10 g. of adipic dihydrazide were added slowly. The pH was adjusted to 4.7 by the dropwise addition of 1N HCl then 1.25 g of 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide were added and the pH was readjusted to 4.7 with 1N HCl. The reaction mixture was then stirred for 20 hours at 23-25°C. The crude reaction product was purified by gel

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filtration chromatography on a 4.5 x 55 ml column of Sepahadex® G-25. The column was eluted with phosphate buffered saline (PBS, 0.01 M sodium phosphate, 0.15 M sodium chloride, pH 7.4) and the void volume fractions were saved. The hydrazide-Ficoll was then dialyzed against water and lyophilized. The final product contained about 150 moles of hydrazide/mole of Ficoll.

The hydrazide Ficoll (0.25 g, 0.004 mmole Ficoll 0.23 meq. hydrazide) was dissolved in 10 ml water and adjusted to pH 5.0. Hematoporphyrin (0.069 g, 0.12 mmole) was added followed by 1-ethyl-3-(3-dimethyl-aminopropyl)(0.44 g, 2.3 mmole), and the reaction mixture was stirred overnight. The solution was then dialyzed against Na₂CO₃ solution, pH 9.0 for approximately 5 days. The solution was then lyophilized and the brownish powder stored at 0°C.

12. EXAMPLE: ATTACHMENT OF HEMATOPORPHYRIN DIHYDRAZIDE TO ANTIBODY

The specific mouse monoclonal antibody utilized in the following experiments was CYT-021. The antibody is a monoclonal IgG which is specific for a glycoprotein antigen on human cytotoxic/suppressor T-lymphocytes.

12.1. PREPARATION OF ANTIBODY-HEMATOPORPHYRIN DIHYDRAZIDE CONJUGATES

The hematoporphyrin dihydrazide was attached to the CYT-021 antibody for use in photoradiation treatment of cellular disorders. Antibody-hematoporphyrin dihydrazide conjugates were prepared according to one method of the instant invention by first oxidizing the carbohydrate moiety of the antibody molecule. The carbohydrate moiety of CYT-021 antibody was oxidized by reacting approximately 1 mg/ml of antibody in phosphate buffered saline (PBS) with 110 µl of

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100 mM sodium metaperiodate (NaIO_4) at pH 6 (to give a final concentration of 10 mM NaIO_4) for 1 hour on ice in the dark.

5 Excess NaIO_4 was removed by passing the solution through a 10 ml Sephadex® G-50 column (Pharmacia Fine Chemicals, Piscataway, NJ) which had been prewashed with 1 ml of a 10% bovine serum albumin (BSA) solution in phosphate buffered saline (PBS). The protein was eluted with PBS, pH 6 and 1 ml fractions were collected, OD_{280} determined and the
10 protein fractions were pooled. The oxidized CYT-021 antibody was then attached to hematoporphyrin dihydrazide as described below and the CYT-021 antibody-hematoporphyrin dihydrazide conjugate either used immediately or stored frozen at -20°C protected from light.

15 Hematoporphyrin dihydrazide was coupled to oxidized CYT-021 antibody as follows. A solution of hematoporphyrin dihydrazide was prepared by dissolving 5 mg of hematoporphyrin dihydrazide in 50 μl of N,N-dimethylformamide (DMF). The
20 concentration of hematoporphyrin dihydrazide was adjusted to 1 mg/ml by the addition of 5 ml of deionized water.

25 500 μl of oxidized CYT-021 antibody, in PBS, pH 6.0 was incubated with an equal volume of the hematoporphyrin dihydrazide solution for 30 minutes in the dark, at room temperature. To reduce the hydrazone product, sodium
30 cyanoborohydride (NaCNBH_3) was added to a final concentration of 10 mM and the reaction mixture was incubated overnight at room temperature.

35 Free hematoporphyrin dihydrazide, in PBS, pH 6.0, was removed by passing the reaction mixture over a 10 ml Sephadex® G-50 column (Pharmacia Fine Chemicals, Piscataway, NJ) which had been pre-washed with 1 ml of a 10% solution of BSA in PBS. The protein was eluted with PBS, pH 6 and 1 ml

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fractions were collected, and protein fractions were pooled. The amount of hematoporphyrin dihydrazide bound to CYT-021 antibody was determined spectrophotometrically by using the extinction coefficients for protein at 280 nm and hematoporphyrin dihydrazide at 374 nm, and ranged from 4 to 8 moles/mole antibody.

12.2. CYTOTOXICITY ASSAY USING THE ANTIBODY-HEMATOPORPHYRIN DIHYDRAZIDE CONJUGATE

In vitro cytotoxicity assays were carried out in 96 well U-bottom Costar tissue culture plates (Costar, Cambridge, MA). The target cells (MOLT-4, ATCC CRL 1582), were obtained from American Type Culture Collection, Rockville, MD as were control cells (Raji, ATCC CCL 86). The MOLT-4 cell line is a T-cell line derived from the human peripheral blood of a patient with acute lymphoblastic leukemia. The Raji cell line is a lymphoblastic-like cell line derived from a patient with Burkitt's lymphoma. The cells were washed 2 times with RPMI 1640 culture medium (10% Fetal Calf Serum, 2% Glutamine, 1% Penicillin-Streptomycin) (M.A. Bioproducts, Walkersville, MD) and diluted to a concentration of 10^6 cells/ml. The CYT-021 antibody-hematoporphyrin dihydrazide conjugate was filtered through a 0.45 micron syringe filter (Gelman, Ann Arbor, MI) and 1:10 dilutions of the conjugate were prepared.

One ml of cells were mixed with 1 ml of the conjugate or appropriate controls and were incubated in the dark for 2 hours in a humidified 37°C, 5% CO₂ incubator. The cells were washed three times with RPMI 1640 culture medium (maintaining dark conditions) and the pellets resuspended in RPMI 1640 medium. Aliquots of 10^4 or 10^5 cells (see Table IX) (in a volume of 100 μ l) were plated in triplicate on each microtiter plate and duplicate plates were prepared. One set

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of microtiter plates were incubated in the dark for 24 hours as described above. The other set of microtiter plates was then incubated overnight as above while exposed to a standard fluorescent light source.

5 Viability of cells was then determined by measuring ^3H -thymidine (New England Nuclear, Boston, MA) uptake. The microtiter plates were centrifuged in a TJ-6 centrifuge (Beckman Instruments, Palo Alto, CA) for 5 minutes at 1500 rpm. The supernatants were removed and 100 ul of RPMI
10 culture medium containing 1 uCi of ^3H -thymidine (New England Nuclear, Boston, MA) was added to each well. The cells were incubated for at least 4 hours in the CO_2 incubator before harvesting onto filter paper using a MASH (Skatron Inc., Sterling, VA). The filter paper was dissolved in 3 ml
15 Aquasol-2 (New England Nuclear, Boston, MA) and counted on a LKB 1212 liquid scintillation counter.

20

TABLE IX

CYTOTOXICITY OF ANTIBODY-HEMATOPORPHYRIN
DIHYDRAZIDE CONJUGATE (Ab-Hd)

	<u>Cell Type</u>	<u>Ab-Hd mg/ml</u>	<u>% Cell Killing</u>	
			<u>Dark</u>	<u>Light</u>
25	Molt-4	0	0	0
		0.05	15	99
		0.005	14	95
	Raji	0	0	3
		0.05	0	7

30

As illustrated in Table IX, the percent of killing of target cells incubated with the Ab-Hd conjugate and exposed to light was close to 100 percent. Control cells incubated with the

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CYT-021 Antibody-hematoporphyrin-dihydrazide conjugate and kept in the dark incorporated maximum amounts of ^3H -thymidine and no significant mortality was observed.

5

13. EXAMPLES: PREPARATION OF ANTIBODY
CONJUGATES VIA SULFHYDRYL ATTACHMENT

13.1. HYDRAZIDE-FICOLL

10 Ficoll 70 was obtained from Pharmacia Fine Chemicals, Inc. (Piscataway, NJ). The hydrazide-ficoll was prepared by the method described in Section 11.5.

13.2. (4-iodoacetyl)-aminobenzoichydrazide-ficoll

15 Thirty milligrams of N-succinimidyl-(4-iodoacetyl)-aminobenzoate (SIAB, Pierce Chemical Co., Rockford, IL) were dissolved in 3 ml of acetonitrile. Six
20 0.48 ml aliquots of the SIAB solution were added to 20 ml of a 5 mg/ml solution of hydrazide-Ficoll (prepared as described in Section 13.1) dissolved in 0.1 M sodium phosphate buffer, pH 7.0, at 30 minute intervals. Before the second addition
25 of SIAB solution, 5 ml of tetrahydrofuran were added to the hydrazide-Ficoll to clarify the solution. The reaction mixture was then stirred for 20 hours at 23-25°C. The organic solvents were removed by bubbling N_2 gas through the
30 reaction mixture and the resultant cloudy solution was clarified by centrifugation. The clear supernatant was lyophilized. Excess SIAB was removed by extracting the dry powder with tetrahydrofuran and the excess solvent was removed by evaporation under reduced pressure. The dry powder was dissolved in 5 ml of water and dialyzed for 4 hours at 4°C. The (4-iodoacetyl)-aminobenzoichydrazide-Ficoll was stored frozen at -70°C and contained about 16 iodoacetyl groups per mole of hydrazide-Ficoll.

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13.3. (4-iodoacetyl)-aminobenzoic hydrazide
ficoll-acetylthio-IgG

One milligram of mouse anti-N. gonorrhoeae monoclonal IgG was diluted into 0.25 ml of PBS, pH 7.4. The IgG solution was then reduced with 5 mM dithiothreitol for 30 minutes at 23-25°C. The reduced antibody was then passed over 1x19 cm Sephadex® G-50 column and eluted with 0.1 M tris(hydroxymethyl)-aminomethane buffer, pH 8.0, containing 1mM ethylenediaminetetracetic acid (Tris/EDTA). One milligram of (4-iodoacetyl)-aminobenzoic hydrazide-ficoll (prepared as described in Section 13.2) was added to the reduced antibody and the reaction mixture was incubated at 4°C for 16 hours to form an iodoacetyl-aminobenzoic hydrazide-ficoll-acetylthio-IgG conjugate.

This is an example of the preparation of an antibody conjugate to which ¹⁵³Gd metal ion is complexed wherein the conjugate is formed by attachment of a single site linker at plurality of sites introduced at the sulfhydryl groups of antibodies. By analogous mechanisms, a therapeutic agent may be attached to an antibody or antibody fragment.

13.4. MALEIMIDE HYDRAZIDE FICOLL

Twenty-two milligrams of 4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester (SMCC, Sigma Chemical Co., St. Louis, MO) were dissolved in 3 ml of tetrahydrofuran. Six 0.5 ml aliquots of the SMCC solution were added to 20 ml of a 5 mg/ml solution of hydrazide-ficoll (prepared as described in Section 13.1), dissolved in 0.1 M sodium phosphate buffer, pH 6.8, at 30 minute intervals. Two milliliters of tetrahydrofuran were added to the reaction mixture before the second and third

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additions of SMCC solution. The organic solvents were then removed by bubbling N_2 through the reaction mixture and the resultant cloudy solution was clarified by centrifugation. The clear supernatant was lyophilized and the excess SMCC was extracted with tetrahydrofuran. The organic solvent was then removed by evaporation under reduced pressure. The final produce contained about 15 moles of maleimide/mole of Ficoll. This compound can be used in the same way as the (4-iodoacetyl)-aminobenzoic hydrazide-Ficoll prepared by the method of Section 13.2.

14. EXAMPLES: PREPARATION OF ERYTHROSIN DERIVATIVES

The following examples illustrate the novel derivatives of erythrosin which can be used as photoactivatable cytolytic agents (or photosensitizers) for in vivo therapy when specifically attached to an antibody molecule.

14.1. PREPARATION OF 5-ERYTHROSIN ISOTHIOCYANATE

Erythrosin 5-isothiocyanate (Aldrich Chemicals, Milwaukee, WI) was prepared according to the procedure of Moore and Garland (Biochemical Society Transactions, 1979, 7: 945-946) with certain modifications. The conversion of 5-amino erythrosin into the isothiocyanate was performed as follows: thiophosgene (0.34 g, 0.227 ml, 3.0 mmole) was added dropwise to a suspension of 5-amino erythrosin (1.2 g, 1.4 mmole) in toluene (20 ml) at 0°C. After addition of the thiophosgene over 10 minutes, the solution was warmed to room temperature, then heated to reflux for 2 hours. Evaporation of the solvent gave a red solid which showed one major component by the technique of thin layer chromatography using a developing solvent composed of (silica gel, ethyl acetate: pyridine: acetic acid (E:P:A) (50:1:1; v:v:v). The weight of

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the product was 1.33 g, and the product showed a strong isothiocyanate band in the infrared spectrum at 2045 cm^{-1} .

14.2. PREPARATION OF 5-ERYTHROSIN THIOSEMICARBAZIDE

5 Anhydrous hydrazine (0.007 g, 0.007 ml, 0.22 mmole) was added to a solution of 5-erythrosin isothiocyanate (0.1 g, 0.11 mmole) in tetrahydrofuran (5 ml). The solution was stirred overnight and the precipitated solid filtered to give red crystals which showed absence of the thiocyanate group (2040 cm^{-1}) in the infrared spectrum..

14.3. PREPARATION OF N-(2-AMINOETHYL) ERYTHROSIN 5-THIOUREA

15 Erythrosin 5-isothiocyanate (0.45 g, 0.5 mmole) was dissolved in 20 ml of tetrahydrofuran and cooled to 0°C . Ethylene diamine (0.3 g, 0.334 ml, 5.0 mmole) was added dropwise over ten minutes and the reaction stirred at room temperature overnight. Filtration of the solid red precipitate was followed by washing the tetrahydrofuran and drying at 80°C under vacuum. The red solid weighed 0.52 g and had no isothiocyanate peak in the infrared at approximately 2040 cm^{-1} . This product was suspended in 5% HCl filtered and dried to give a red solid weighing 0.34 g.

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14.4. PREPARATION OF ERYTHROSIN-5-HYDRAZIDE

30 Solid sodium nitrite (0.01 g, 0.15 mmole) was added to a solution of erythrosin-5-amine (0.1 g, 0.11 mmole) (prepared by the method of Moore and Garland, 1979, Biochemical Society Transactions 7: 945-946) in 10 ml of 6N HCl at 0°C . The diazotizing solution was stirred for 3 hours at 0°C and a solution of stannous chloride (0.1 g, 0.44 mole) in 2 ml of 3 M HCl was added. The red solution was stirred

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at room temperature for 3 hours and then filtered. The deep red precipitate was washed with water, and dried. The red product showed binding to aldehydic oxidized Sepharose beads at pH 8.8 but no binding to non-oxidized non-aldehydic Sepharose, indicating the presence of a hydrazine group.

14.5. PREPARATION OF FICOLL-HYDRAZIDE-ERYTHROSIN

Ficoll hydrazide (0.25 g 0.004 mmole, 0.23 meq. hydrazide) was dissolved in 40 ml of 50% ethanol: water. Erythrosin isothiocyanate (0.022 g, 0.025 mmole) was added and the solution stirred overnight. Dialysis against Na_2CO_3 , pH 9.0 for 5 days, followed by lyophilization gave a pink powder which was water-soluble and displayed a maximum wavelength of about 524 nm in pH 9 buffer.

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

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WHAT IS CLAIMED IS:

5 1. An antibody-therapeutic agent conjugate,
comprising: a therapeutic agent capable of acting as a
photothermolytic agent, as a photosensitizer or as a
substrate activator to mediate cytotoxic effects
attached through a covalent bond to an antibody or
antibody fragment to form an antibody-therapeutic agent
10 conjugate having substantially the same immunoreactivity
and immunospecificity as the unconjugated antibody or
antibody fragment, wherein the covalent bond is
selectively formed at a site located outside the antigen
binding region of the antibody or antibody fragment.

15 2. The antibody-therapeutic agent conjugate
according to claim 1, in which: the therapeutic agent
is attached via a covalent bond to a carbohydrate moiety
of an oxidized antibody or antibody fragment, wherein
the covalent bond is an imine, enamine, hydrazone,
20 oxime, phenylhydrazone, semicarbazone,
thiosemicarbazone, or a reduced form thereof.

25 3. The antibody-therapeutic agent conjugate
according to claim 2, wherein the antibody fragment is
selected from the group consisting of Fab fragments,
(Fab')₂ fragments and half antibody molecules.

30 4. The antibody-therapeutic agent conjugate
according to claim 1, wherein the antibody or antibody
fragment is a monoclonal antibody or a monoclonal
antibody fragment.

35 5. The antibody-therapeutic agent conjugate
according to claim 1, in which a therapeutic agent is

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attached via a covalent bond to a sulfur atom of a reduced antibody or Fab' fragment.

5 6. The antibody-therapeutic agent conjugate according to claim 5, wherein the antibody or Fab' fragment is a monoclonal antibody or monoclonal antibody Fab' fragment.

10 7. The antibody-therapeutic agent conjugate according to claim 1, wherein the therapeutic agent capable of acting as a photosensitizer to mediate cytotoxic effects is selected from the group consisting of photoporphyrin, deuteroporphyrin, hematoporphyrin, rose bengal, an acridine, a thiazine, a xanthene, an anthraquinone, an azine, a flavin, methylene blue, 15 eosin, erythrosin and psoralin.

20 8. The antibody-therapeutic agent conjugate according to claim 1, wherein the therapeutic agent capable of acting as a substrate activator to mediate cytotoxic effects is selected from the group consisting of glucose oxidase, galactose oxidase and xanthene oxidase.

25 9. An antibody-therapeutic agent conjugate, comprising a therapeutic agent capable of acting as a photothermolytic agent, as a photosensitizer, or as a substrate activator to mediate cytotoxic effects covalently attached to a linker which is attached through a covalent bond to an antibody or antibody 30 fragment to form an antibody-therapeutic agent conjugate having substantially the same immunoreactivity and immunospecificity as the unconjugated antibody or antibody fragment, wherein the covalent bond is

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selectively formed at a site located outside the antigen binding region of the antibody or antibody fragment.

5 10. The antibody-therapeutic agent conjugate according to claim 9, in which: the therapeutic agent is attached via a covalent bond to a carbohydrate moiety of an oxidized antibody or antibody fragment, wherein the covalent bond is an imine, enamine, hydrazone, oxime, phenylhydrazone, semicarbazone, thiosemicarbazone, or a reduced form thereof.

10 11. The antibody-therapeutic agent conjugate according to claim 9, wherein the antibody fragment is selected from the group consisting of Fab fragments, (Fab')₂ fragments and half antibody molecules.

15 12. The antibody therapeutic agent conjugate according to claim 9, wherein the antibody or antibody fragment is a monoclonal antibody or a monoclonal antibody fragment.

20 13. The antibody therapeutic agent conjugate according to claim 9, in which a therapeutic agent is attached via a covalent bond to a sulfur atom of a reduced antibody or Fab' fragment.

25 14. The antibody therapeutic agent conjugate according to claim 9, wherein the antibody or Fab' fragment is a monoclonal antibody or monoclonal antibody Fab' fragment.

30 15. The antibody therapeutic agent conjugate according to claim 9, wherein the therapeutic agent capable of acting as a photosensitizer to mediate cytotoxic effects is selected from the group consisting

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of photoporphyrin, deuteroporphyrin, hematoporphyrin, rose bengal, an acridine, a thiazine, a xanthene, an anthraquinone, an azine, a flavin, methylene blue, eosin, erythrosin and psoralin.

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16. The antibody therapeutic agent conjugate according to claim 9, wherein the therapeutic agent capable of acting as a substrate activator to mediate cytotoxic effects is selected from the group consisting of glucose oxidase, galactose oxidase and xanthene oxidase.

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17. The antibody therapeutic agent conjugate according to claim 9, wherein the linker is a branched linker.

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18. The antibody therapeutic agent conjugate according to claim 9, wherein the linker is susceptible to cleavage by activated complement or by an enzyme having proteolytic activity.

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19. An antibody-linker intermediate, comprising: a branched linker attached through a covalent bond to an antibody or antibody fragment to form an antibody-linker intermediate having substantially the same immunoreactivity and immunospecificity as the unconjugated antibody or antibody fragment, wherein the covalent bond is selectively formed at a site located outside the antigen binding region of the antibody or antibody fragment.

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20. The antibody-linker intermediate according to claim 19, in which a branched linker is attached via a covalent bond to a carbohydrate moiety of an oxidized antibody or antibody fragment, wherein the

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covalent bond is an imine, enamine, hydrazone, oxime, phenylhydrazone, semicarbazone, thiosemicarbazone, or a reduced form thereof.

5 21. The antibody-linker intermediate according to claim 20, wherein the antibody fragment is selected from the group consisting of Fab fragments, (Fab')₂ fragments and half antibody molecules.

10 22. The antibody-linker intermediate according to claim 20, wherein the antibody or antibody fragment is a monoclonal antibody or a monoclonal antibody fragment.

15 23. The antibody-linker intermediate according to claim 19, in which a branched linker is covalently attached to a sulfur atom of a reduced antibody or Fab' fragment.

20 24. The antibody-linker intermediate according to claim 23, wherein the antibody or Fab' fragment is a monoclonal antibody or monoclonal antibody Fab' fragment.

25 25. The antibody-linker intermediate according to claim 19, wherein the branched linker is susceptible to cleavage by activated complement or by an enzyme having proteolytic activity.

30 26. An antibody-therapeutic agent conjugate, comprising: a therapeutic agent covalently attached to a branched linker which is attached through a covalent bond to an antibody or antibody fragment to form an antibody-therapeutic agent conjugate having substantially the same immunoreactivity and

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immunospecificity as the unconjugated antibody or antibody fragment, wherein the covalent bond is selectively formed at a site located outside the antigen binding region of the antibody or antibody fragment.

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27. The antibody-therapeutic agent conjugate according to claim 26, in which a therapeutic agent is covalently attached to a branched linker which is attached via a covalent bond to a carbohydrate moiety of an oxidized antibody or antibody fragment, wherein the
10 covalent bond is an imine, enamine, hydrazone, oxime, phenylhydrazone, semicarbazone, thiosemicarbazone, or a reduced form thereof.

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28. The antibody-therapeutic agent conjugate according to claim 26, wherein the antibody fragment is selected from the group consisting of Fab fragments, (Fab')₂ fragments and half antibody molecules.

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29. The antibody-therapeutic agent conjugate according to claim 26, wherein the antibody or antibody fragment is a monoclonal antibody or a monoclonal antibody fragment.

25
30. The antibody-therapeutic agent conjugate according to claim 26, in which a therapeutic agent is covalently attached to a linker which is covalently attached to a sulfur atom of a reduced antibody or Fab' fragment.

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31. The antibody-therapeutic agent conjugate according to claim 30, wherein the antibody or Fab' fragment is a monoclonal antibody or monoclonal antibody Fab' fragment.

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32. The antibody-therapeutic agent conjugate according to claim 26, wherein the branched linker comprises a spacer element and a cleavable element.

5 33. The antibody-therapeutic agent conjugate according to claim 32, wherein the cleavable element is susceptible to cleavage by complement or by an enzyme having proteolytic activity.

10 34. A linker-therapeutic agent intermediate, comprising a linker covalently attached to a therapeutic agent, in which the linker contains a reactive group capable of selective covalent attachment to an antibody or antibody fragment at a site outside the antigen binding region of the antibody or antibody fragment to
15 form an antibody-therapeutic agent conjugate having substantially the same immunoreactivity and immunospecificity as the unconjugated antibody or antibody fragment.

20 35. The linker-therapeutic agent according to claim 34, in which the reactive group is selected from the group consisting of primary amine, secondary amine, hydrazine, hydrazide, hydroxylamine, phenylhydrazine, semicarbazide and thiosemicarbazide groups, and said
25 reactive group is capable of covalent attachment to a carbohydrate moiety of an oxidized antibody or antibody fragment.

30 36. The linker-therapeutic agent intermediate according to claim 34, in which the reactive group is selected from the group consisting of haloalkyl groups, p-mercuribenzoate groups, and groups capable of Michael-type addition reactions and said reactive group

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is capable of covalent attachment to a sulfhydryl group of a reduced antibody or antibody Fab' fragment.

5 37. The linker-therapeutic agent intermediate according to claim 34, 35 or 36, wherein the linker is a branched linker.

10 38. The linker-therapeutic agent intermediate according to claim 34, in which the therapeutic agent is a photothermolytic agent, a photosensitizer or a substrate activator capable of mediating cytotoxic effects.

15 39. The linker-therapeutic agent intermediate according to claim 38, wherein the photosensitizer is selected from the groups consisting of protoporphyrin, deuteroporphyrin, hematoporphyrin, rose bengal, an acridine, a thiazine, a xanthene, an anthraquinone, an azine, a flavin, methylene blue, eosin, erythrosin and psoralin.

20 40. The linker-therapeutic agent intermediate according to claim 38, wherein the therapeutic agent capable of acting as a substrate activator to mediate cytotoxic effects is selected from the group consisting of glucose oxidase, galactose oxidase and xanthene oxidase.

25 41. A method for therapeutic treatment of cellular disorders, comprising: administering a therapeutically effective amount of antibody-therapeutic agent conjugate according to claim 1, 2, 6, 9, 10, 26, 27, or 30, said antibody-therapeutic agent conjugate being immunoreactive with and immunospecific for a target site associated with said cellular disorder

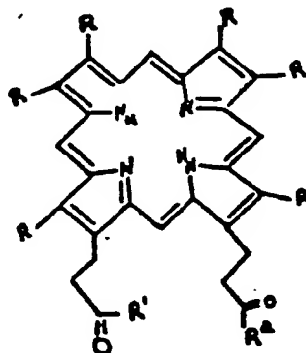
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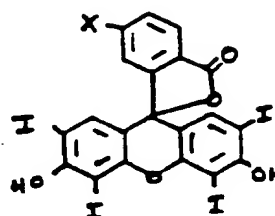
substantially non-immunoreactive and non-immunospecific
for tissue not associated with said cellular disorder.

42. A compound of the formula:



wherein R is selected from the group consisting of an
alkyl containing from 1 to 3 carbon atoms, a hydroxy
alkyl containing from 1 to 3 carbon atoms, a carboxyl,
an alkyl carboxyl in which the alkyl contains from 1 to
3 carbon atoms, a vinyl and an H, and R^1 and R^2 are
 $NHNH_2$, or R^1 is OH and R^2 is $NHNH_2$, or R^1 is $NHNH_2$ and
 R^2 is OH.

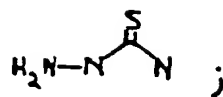
43. A compound of the formula:



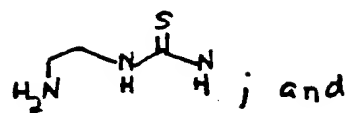
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wherein X is selected from the group consisting of

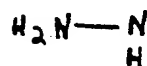
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FIG. 1

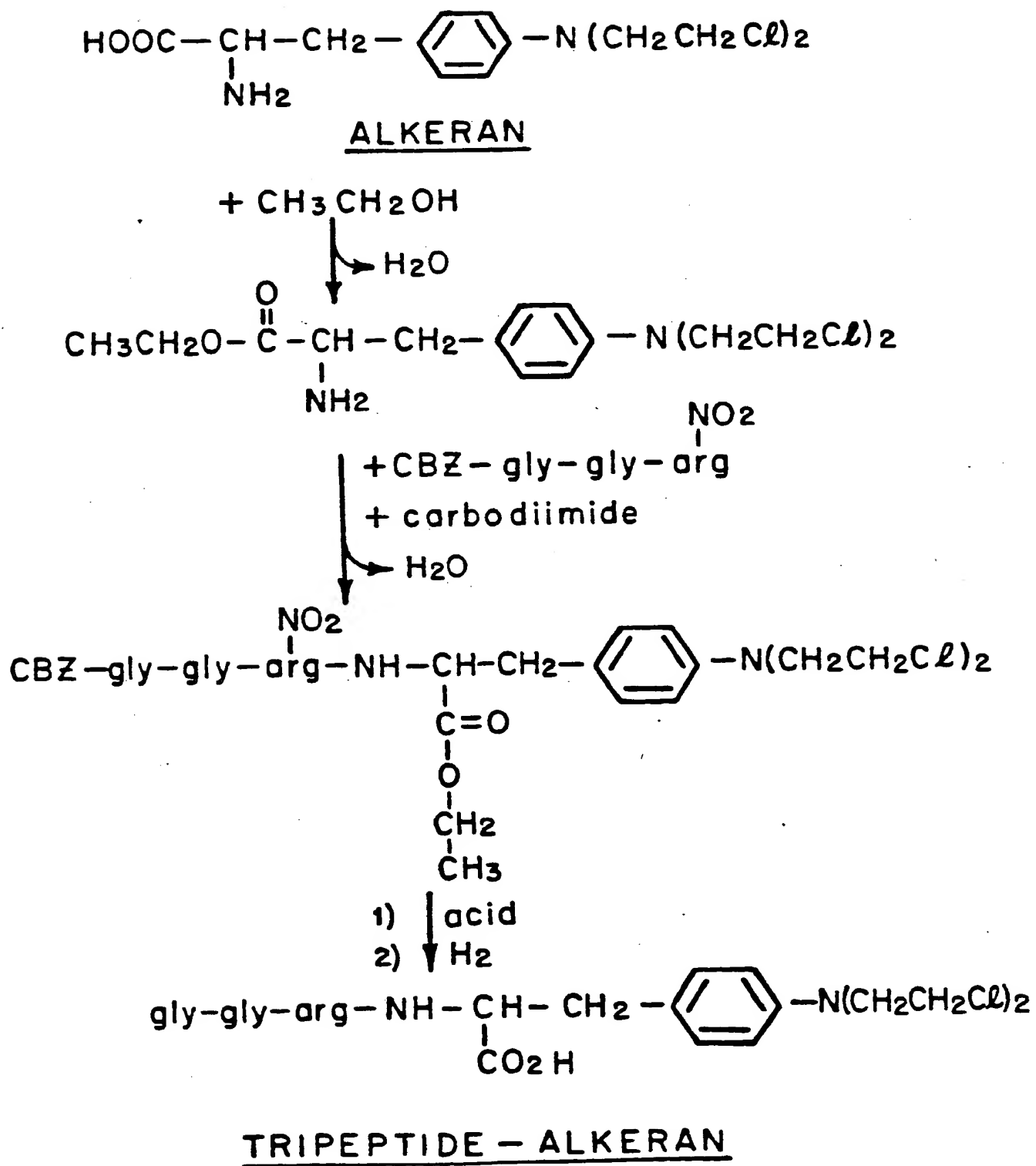
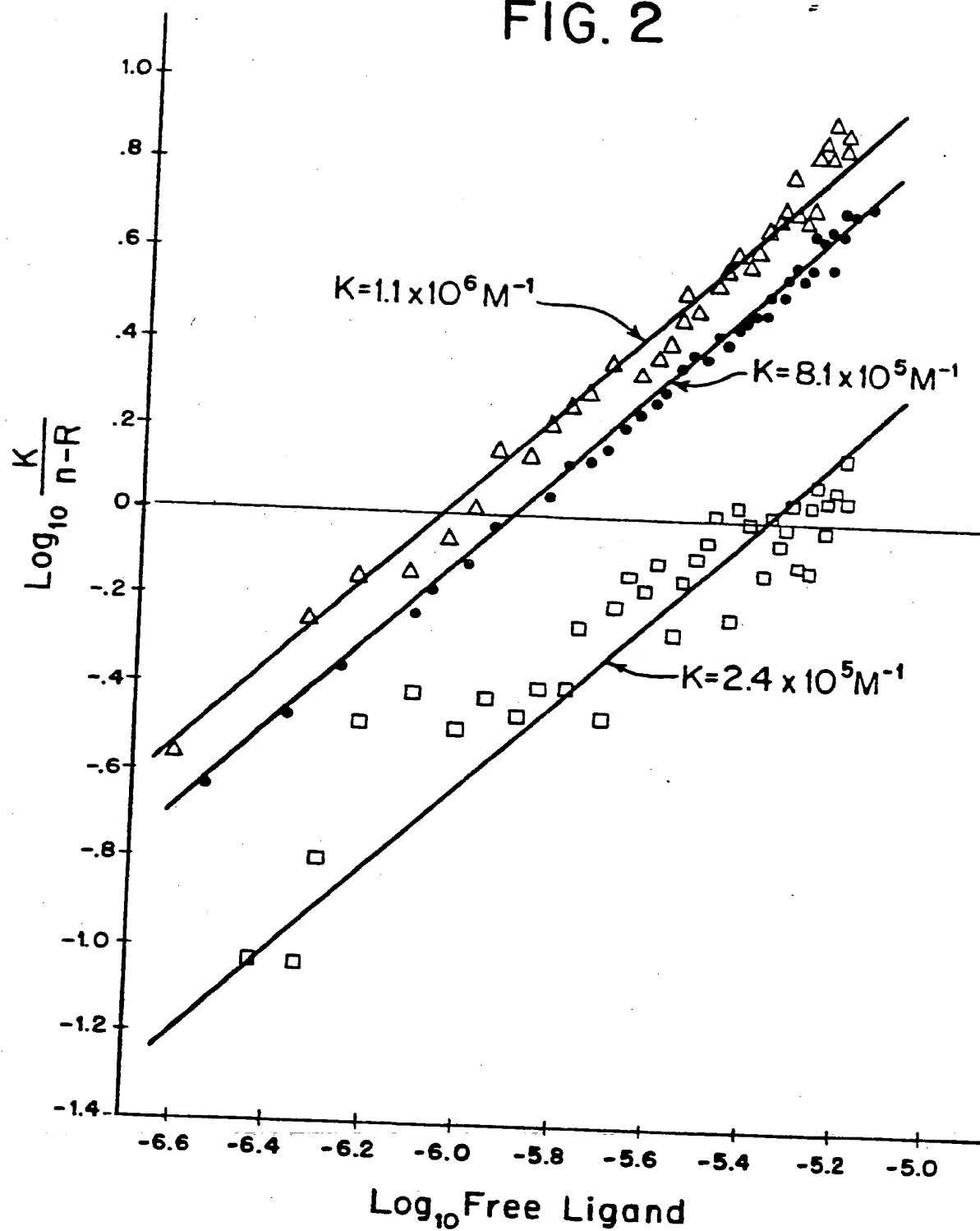


FIG. 2



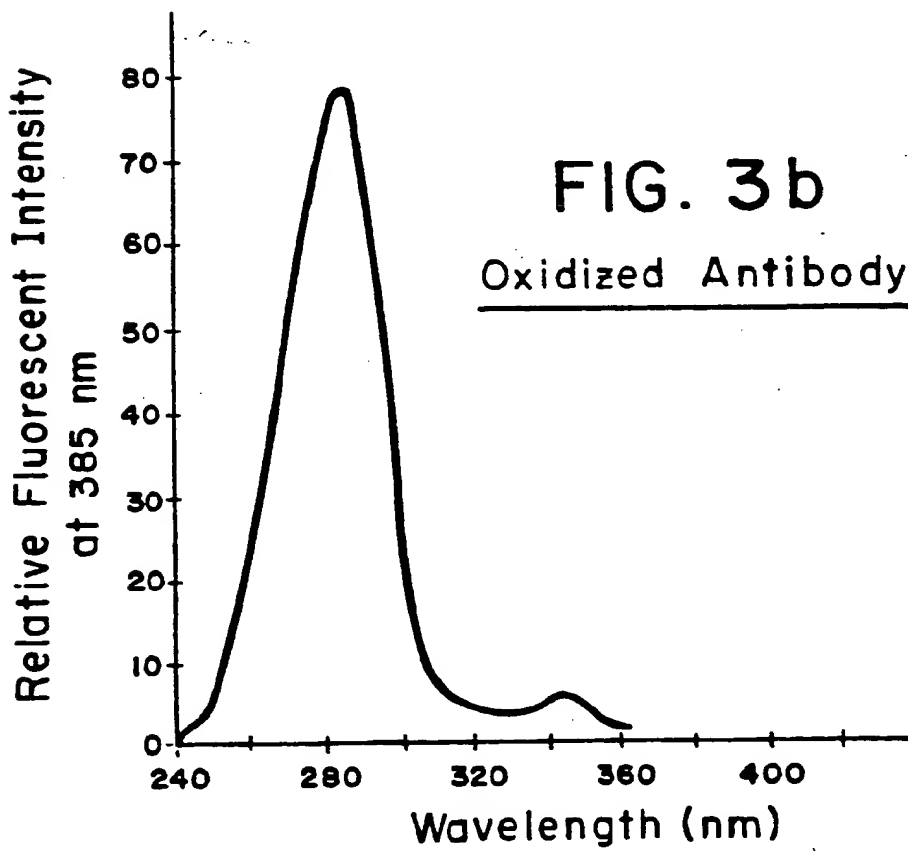
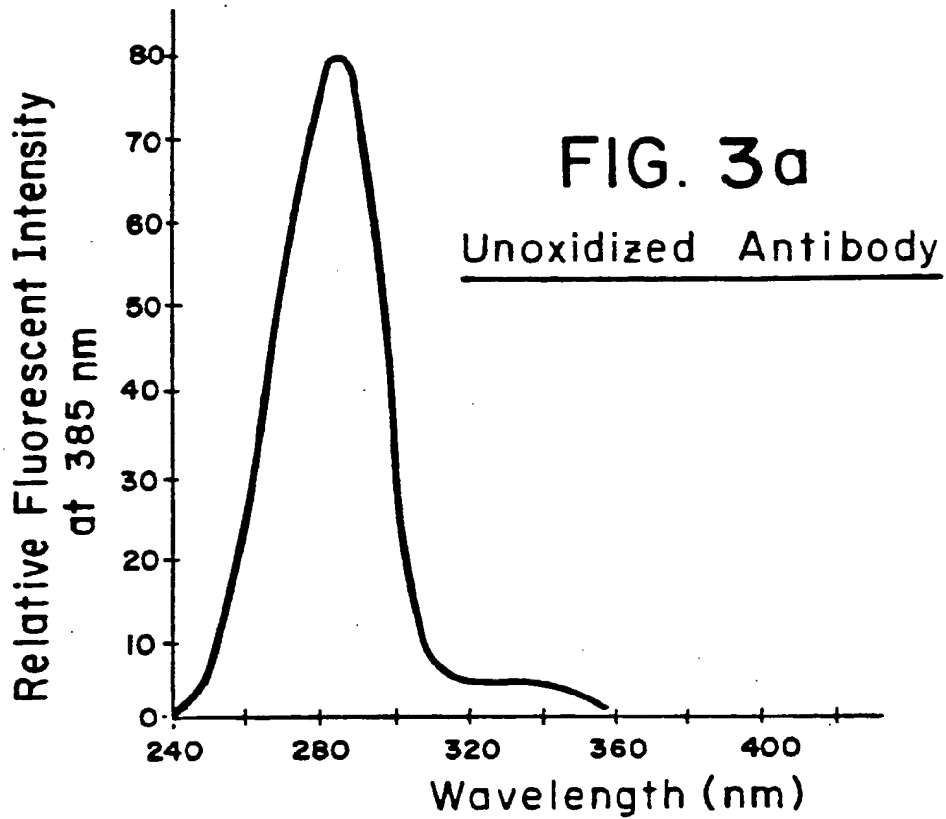
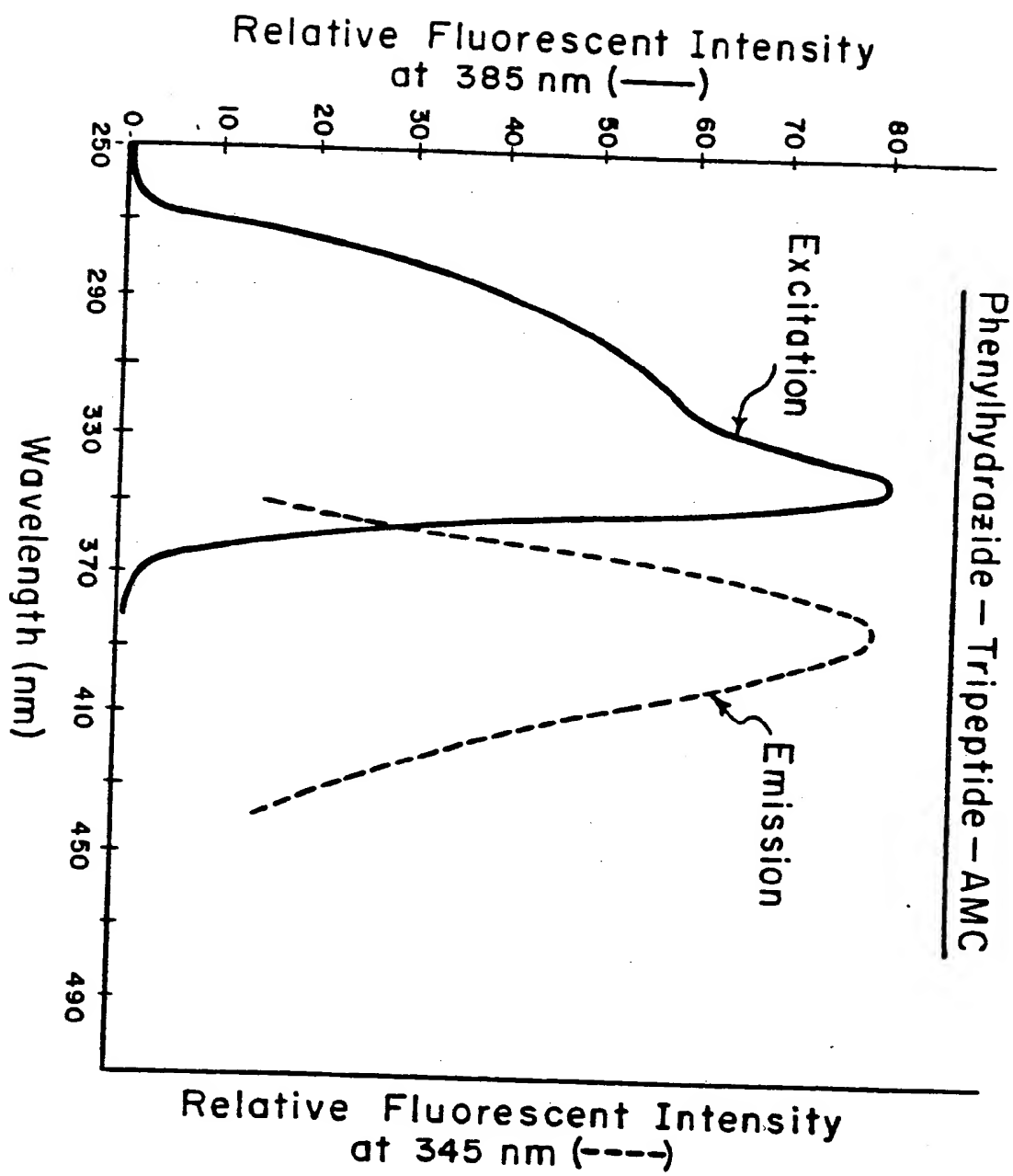


FIG. 4



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FIG. 5

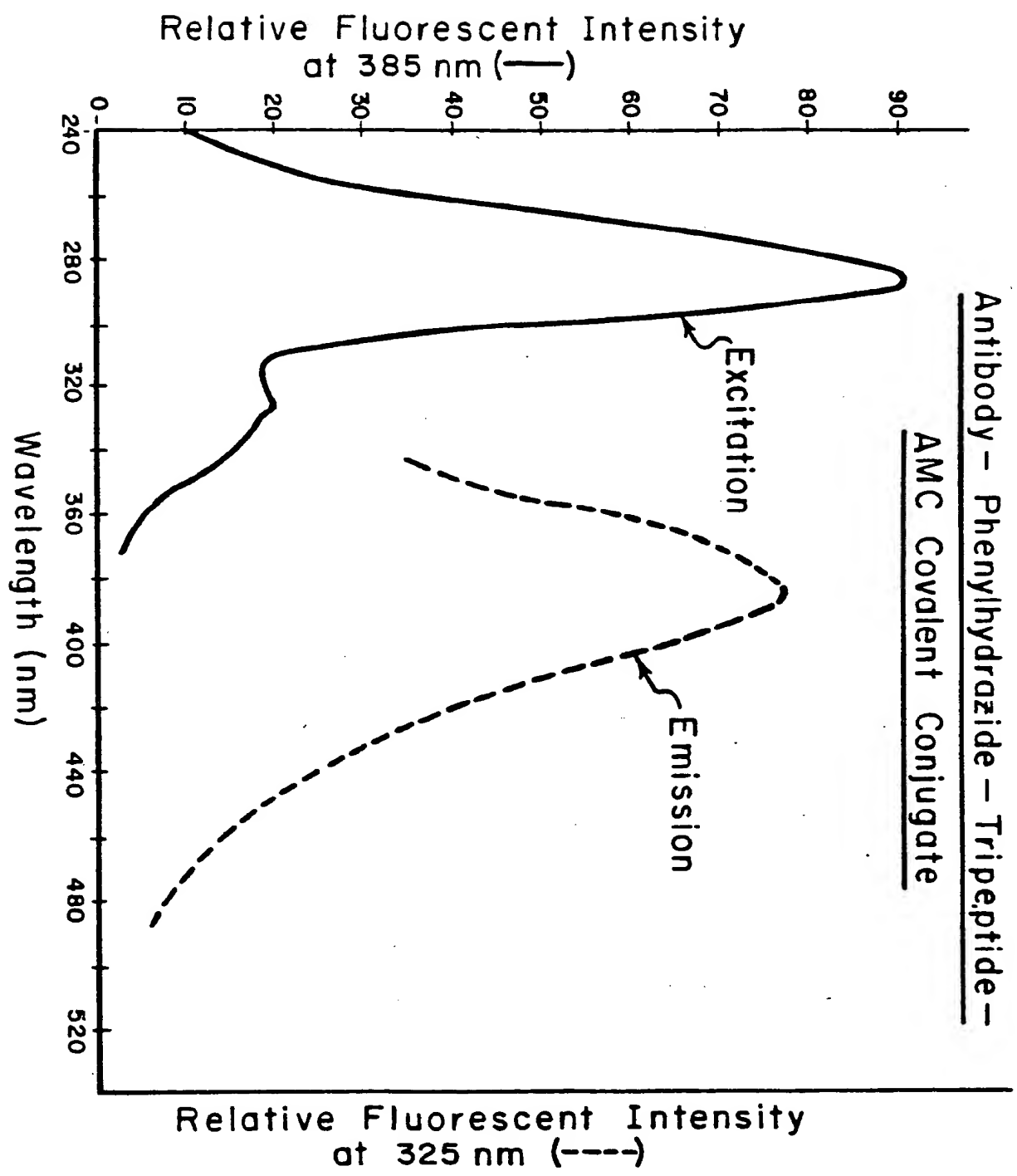
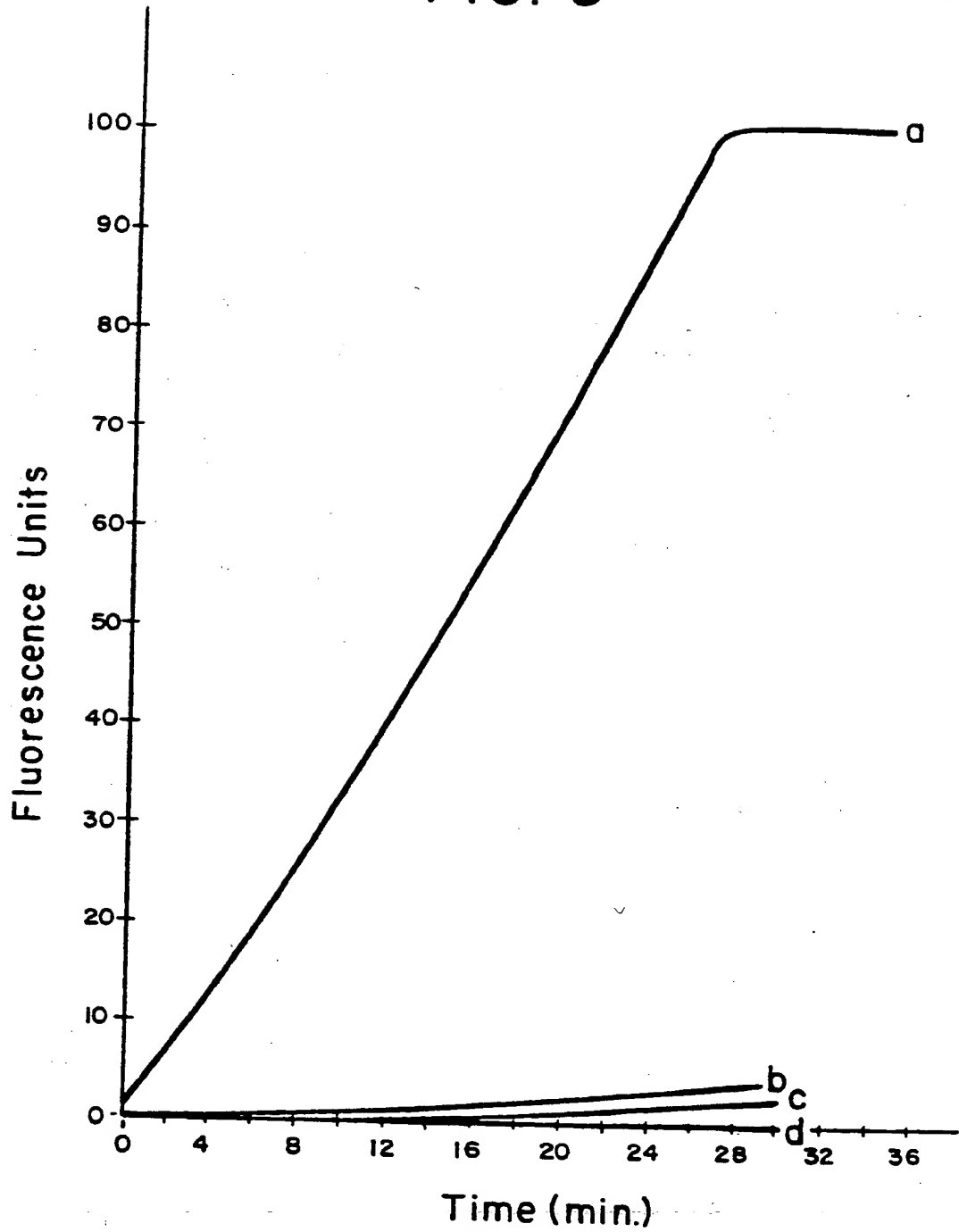


FIG. 6



(19)



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(71) Applicant: **CYTOGEN CORPORATION, 201 College
Road East Forrester Research Center, Princeton New
Jersey 08540 (US)**

(72) Inventor: **Goers, John Walter, 5200 Dolores Street,
Atascadero California (US)**
Inventor: **Lee, Chyl, 1347 Ute Road, New Brunswick New
Jersey (US)**
Inventor: **Siegel, Richard Charles, 117 Whiton Road,
Neschanic Station New Jersey (US)**
Inventor: **McKearn, Thomas Joseph, R.D. No. 3 Box 119,
New Hope Pennsylvania (US)**
Inventor: **King, Hurley Dalton, 1688-B Bluebird Drive,
Yardley Pennsylvania (US)**
Inventor: **Coughlin, Daniel James, 37-07 Hunters,
Plainsboro New Jersey (US)**
Inventor: **Rodwell, John Dennis, 430 Ramsey Road,
Yardley Pennsylvania (US)**
Inventor: **Alvarez, Vernon Leon, 187 Rice Drive,
Morrisville Pennsylvania (US)**

(74) Representative: **Warcoln, Jacques et al, Cabinet
Régimbeau 26, avenue Kléber, F-75116 Paris (FR)**

(54) **Antibody-therapeutic agent conjugates.**

(57) This invention relates to antibody-therapeutic agent conjugates having a therapeutic agent covalently attached to an antibody or antibody fragment. Also described are methods for intermediates in the preparation of antibody conjugates. Therapeutic in vivo methods utilizing such antibody-therapeutic agent conjugates are described. Additionally novel photosensitizers suitable for use in preparing antibody-therapeutic agents are also described.

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PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention
shall be considered, for the purposes of subsequent
proceedings, as the European search report

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Application number
EP 85 40 1776

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
Y	EP - A - 0 088 695 (CYTOGEN CORP.) * Whole document *	1-40,42	A 61 K 39/395
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Y	CHEMICAL ABSTRACTS, vol. 82, no. 3, 20th January 1975, page 361, abstract no. 15067u, Columbus, Ohio, US; W.T. SHEARER et al.: "Cytotoxicity with antibody-glucose oxidase conjugates specific for a human colonic cancer and carcinoembryonic antigen", & INT.J. CANCER 1974,		

TECHNICAL FIELDS
SEARCHED (Int. Cl.4)

A 61 K

INCOMPLETE SEARCH

-2-

The Search Division considers that the present European patent application does not comply with
the provisions of the European Patent Convention to such an extent that it is not possible to carry
out a meaningful search into the state of the art on the basis of some of the claims.

Claims searched completely:

Claims searched incompletely:

Claims not searched:

41

Reason for the limitation of the search:

Method for treatment of the human or
animal body by surgery or therapy
(see art. 52(4) of the European Patent
Convention).

Place of search
THE HAGUE

Date of completion of the search
22-03-1988

Examiner
SKELLY

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another
document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or
after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding
document

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CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

- ☐ All claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for all claims.
- ☐ Only part of the claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid, namely claims:
- ☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

X

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirement of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims 1-40: Antibody-therapeutic agent conjugates
2. Claim 42: Novel compound
3. Claim 43: Novel compound

- ☒ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.
- ☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid, namely claims:
- ☐ None of the further search fees has been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims, namely claims:



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PARTIAL EUROPEAN SEARCH REPORT

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Application number

EP 85 40 1776

-2-

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
P, Y	14(4), 539-47 * Abstract * --	8, 16, 40	
	CHEMICAL ABSTRACTS, vol. 102, no. 15, 15th April 1985, page 283, abstract no. 128005x, Columbus, Ohio, US; Ch.K. WAT et al.: "Photosensitizer-protein conjugates: potential use as photoimmunotherapeutic agents", & PROG.CLIN.BIOL.RES. 1984, 170 (Porphyrin localization treatment tumors), 351-9 * Abstract * --	1-7, 9-15, 17-39	
P, Y	CHEMICAL ABSTRACTS, vol. 103, no. 15, 14th October 1985, page 558, abstract 121395m, Columbus, Ohio, US; D. MEW et al.: "Ability of specific monoclonal antibodies and conventional antisera conjugated to hematoporphyrin to label and kill selected cell lines subsequent to light activation", & CANCER RES. 1985, 45(9), 4380-6 * Abstract * --	1-40	TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
A	THE JOURNAL OF IMMUNOLOGY, vol. 125, no. 3, September 1980, page 1201, Williams & Wilkins Co. US; G.W. PHILPOTT et al.: "Selective binding and cytotoxicity of rat basophilic leukemia cells (RBL-1) with immunoglobulin E-biotin and avidin-glucose oxidase conjugates" --		
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DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
A	CHEMICAL ABSTRACTS, vol. 97, no. 7, 16th August 1982, page 224, abstract no. 51193a Columbus, Ohio, US; H. SAKURAI et al.: "Growth inhibition and photooxidative toxicity in the housefly. Musca domestica L. caused by xanthene dye in larval growth medium and after injection" & ENVIRON. ENTOMOL. 1982, 11(2), 467-70 * Abstract *	43	
P, Y	CANCER RESEARCH, vol. 45, September 1985, pages 4380-4386 D. MEW et al.: "Ability of specific monoclonal antibodies and conventional antisera conjugated to hematoporphyrin to label and kill selected cell lines subsequent to light activation" * Whole document *	1-7, 9-15, 17-39	TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
P, A	CHEMICAL ABSTRACTS, vol. 103, no. 5, 5th August 1985, page 491, abstract no. 37259n Columbus, Ohio, US; R. OCAMPO et al.: "Identification of polar porphyrins in oil shale" & J. CHEM. SOC. CHEM. COMMUN. 1985, (4), 198-200 * Abstract *	42	

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